Repair of DNA damage in *Deinococcus radiodurans*

A thesis submitted for the degree of Doctor of Philosophy of Edinburgh University

by

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I hereby confirm that this thesis has been composed by myself and that all the work reported is my own.

David M.Evans

Edinburgh 1984

For Mary-Jane

Abstract

Deinococcus radiodurans is the type species of a group of red-pigmented, radiation-resistant bacteria that comprise one of the eight major groups of the Eubacteria. The repair of DNA lesions in *D.radiodurans* was examined with particular reference to DNA excision repair of ultraviolet light (UV) induced pyrimidine dimers. Repair deficient strains were isolated that were defective in genes designated *uvsC*, *uvsD*, *uvsE*, *uvsF*, *uvsG*, *uvsH* and *rec-1*. The nature of the biophysical and biochemical defects induced by inactivation of these and also the *uvsA* and *mtcA* genes was investigated. A minimum of two mutations is required to eliminate incision of DNA in response to pyrimidine dimers and prevent their subsequent repair. One mutation must be in the *mtcA* gene and one in the *uvsC*, *uvsD* or *uvsE* genes. Three UV endonucleases are postulated to exist in *D.radiodurans*, UV endonuclease α, the product of the mtcA gene, and UV endonuclease β, the product of the *uvsC*, *uvsD* and *uvsE* genes. UV endonuclease β was identified in cell free extracts and partially purified and characterized. The third UV endonuclease termed the HUV endonuclease was also identified in strains lacking UV endonucleases α and β; this enzyme recognises a minor DNA UV photoproduct. All three enzymes are constitutive. UV endonuclease β is a novel type of repair enzyme with an approximate molecular weight of 36000 that acts independently of ATP and which requires manganese ions. This enzyme is inactivated by N-ethylmaleimide. The HUV endonuclease is also small (approximate molecular weight 27000) and ATP independent but is active in EDTA and N-ethylmaleimide. Several other DNA damage specific endonuclease

activities towards other DNA lesions and which also appear to be independent of UV endonucleases α and β are described.

The characteristics of excision repair *via* UV endonucleases α and β *in vivo* varied with respect to (a) the substrate range of the enzymes, (b) the rate of repair of DNA damage and (c) the requirement for a protein synthesised in response to DNA damage to attenuate exonuclease action at repairing regions. UV endonuclease α is postulated to incise DNA in a different manner from UV endonuclease β thereby defining the method of subsequent repair.

Mutations of the *uvsA*, *uvsF* and *uvsG* genes result in an increase in single-strand breaks in response to DNA damage producing uncontrolled DNA degradation. Evidence is presented that these genes have a role in limiting the access of UV endonuclease β to DNA lesions. *uvsF* and *uvsG* are also shown to be linked to the *mtcA* gene.

Mutation of *uvsH* and *rec-1* produces further distinct phenotypes which are discussed. An overall model of excision repair of DNA damage in *D.radiodurans* is presented.

CONTENTS

CHAPTER I

Section 1: The Deinococci

1. Taxonomy of the *Deinococci*

The *Deinococci* are a group of gram-negative, non-sporing, red-pigmented bacteria which until recently were all classified as Micrococci (Fox *et al.*, 1980; Brooks and Murray 1981). Their most notable feature is their extreme resistance to the lethal effects of ionizing and ultraviolet radiation (Brooks and Murray 1981; Moseley 1983). The type species of the new *Deinococcus* genus is *Deinococcus radiodurans* R_1 ATCC 13939 formerly *Micrococcus radiodurans* R_1 ATCC 13939 (Anderson *et al.*, 1956), which is the subject of this thesis. The remaining three species are *D.radiophilus*, formerly *Micrococcus radiophilis* (Lewis 1971, 1973), *D.radiopugnans*, formerly *Micrococcus roseus* ATCC 19172 (Davies *et al.*, 1963) and *D.proteolyticus*, formerly *Micrococcus radioproteolyticus* (Kobatake *et al.*, 1973).

The reclassification of these bacteria into a novel taxon was promoted initially by the unusual structure of the cell envelope which is unlike that found in any other bacterium (Brooks and Murray 1981).The cell envelope of *D.radiodurans*, which appears reasonably representative of those in the other species, is composed of six distinct layers, the outer four of which enclose more than one cell; usually two (Work 1964; Work and Griffiths 1968; Thornley *et al.*, 1965; Schleifer and Kandler 1972; Sleytr *et al.*, 1973; Lancy and Murray 1978). The cell envelopes of *Deinococcus spp.* are also unique in the composition of their peptidoglycan (Thornley *et al.*, 1965; Sleytr *et al.*, 1973; Brooks *et al.*, 1980; Brooks and Murray 1981), their phospholipids (Work and Griffiths 1968; Knivett *et al.*, 1965; Girard 1971; Thompson *et al.*, 1980) and their

lipopolysaccharide (Work and Griffith 1968; Brooks *et al.*, 1980).

The DNA of *Deinococcus spp.* has a high G+C content of 62-70% (Brooks and Murray 1981) and in the case of *D.radiodurans* is unusual in lacking methylated bases (Schein *et al.*, 1972; Storl *et al.*, 1979). However, the DNA of the *Deinococci* is apparently protected from the resident restriction enzymes; Mra I in the case of *D.radiodurans* (Wani *et al.*, 1982), Dra I and Dra II in the case of *D.radiophilus* (Purvis and Moseley 1983; I.Purvis pers.comm.) and restriction enzymes from other genera (M.Mackay pers.comm.) by some novel form of DNA modification (M. Mackay pers.comm.).

DNA homology studies reveal that there is less than 20% homology between species in all instances (Tempest 1978; Brooks *et al.*, 1980) but there are strong similarities between species with respect to their menaquinone systems (Yamada *et al.*, 1977) and their 16S ribonucleic acid sequences (Brooks *et al.*, 1980).

Apart from the resistance of *Deinococcus spp.* to UV radiation (Fig.1) all are resistant to gamma radiation, although the relative survival of the species is difficult to quantify due to the variety of techniques and irradiation conditions used for measurement of survival. Variation in irradiation and culture conditions is known to cause wide variations in survival in *D.radiodurans* (Moseley 1983). However, a typical dose which kills 63% (D_{37} dose) of *D.radiodurans* in aerobic growth medium is 600Krad (Moseley 1983). In addition *Deinococcus spp.* are extremely resistant to desiccation which has been used as an enrichment technique for these bacteria (Saunders and Murray 1979; Murray *et al.*, 1983).

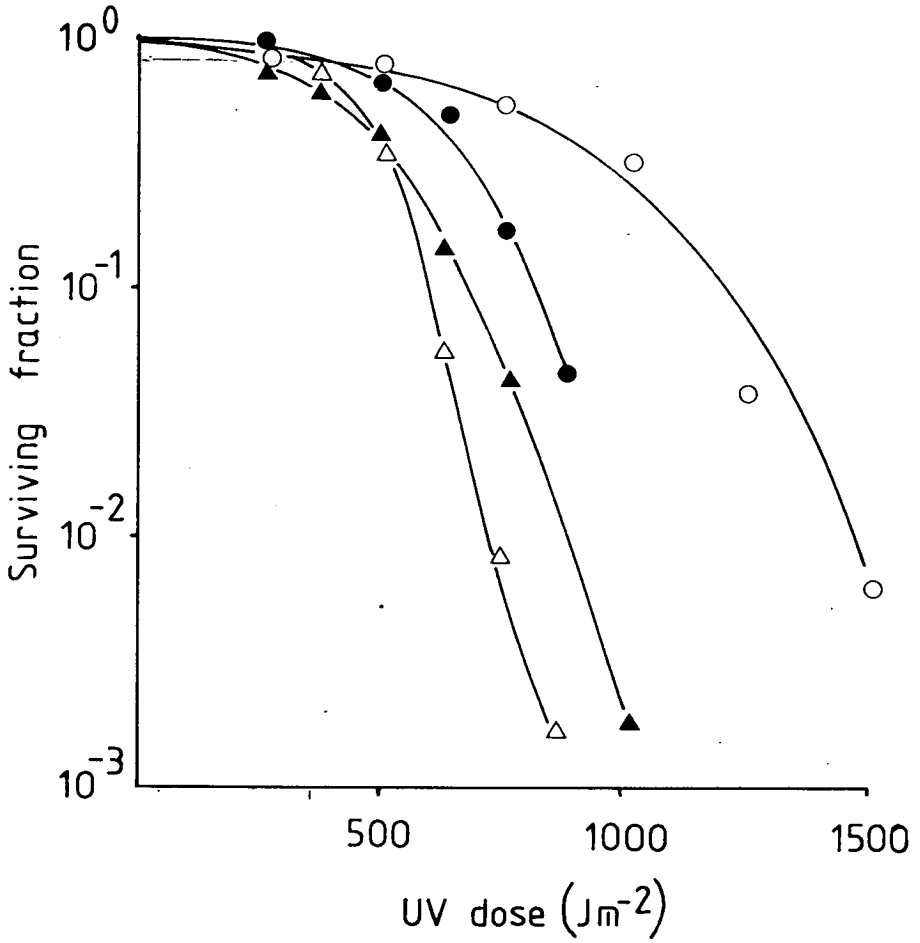


Figure 1

Ultraviolet radiation survival curves of *Deinococcus spp* (data from Tempest 1978).

△ *D.radiodurans*

○ *D.radiophilus*

▲ *D.radiopugnans*

● *D.proteolyticus*

2. Occurrence of *Deinococcus spp.*

The isolation of *D.radiodurans* has been reported on only four occasions. The initial isolation was in Oregon from cans of gamma-irradiated meat (Anderson *et al.*, 1956). The subsequent isolations were from the hides of the live cattle which provided the meat for the canning plant and also the grass and trees in the cattle field (Krabbenhoft *et al.*, 1965), then in Ontario as an aeriel contaminant in a hospital (Murray and Robinow 1958). This strain is called *D.radiodurans* (sark) and has 33% DNA homology with *D.radiodurans* R_1 (Brooks *et al.*, 1980). The most recent isolation was in Japan from gamma-irradiated sawdust used for mushroom culture (Ito *et al.*, 1977).

The remaining species of *Deinococci* have been found in a similar variety of locations. *D.radiophilus* has been isolated on only a single occasion from irradiated Bombay duck (Lewis 1971). *D.radiopugnans* has also been isolated on only a single occasion from irradiated haddock tissue (Davies *et al.*, 1973). Whereas *D.proteolyticus* which was originally isolated from gamma-irradiated llama faeces (Kobatake *et al.*, 1973) has recently been isolated in Japan from animal feed, sewage and surprisingly also from household linen (Ito *et al.*, 1983). It is apparent therefore that *Deinococcus spp.* are widespread and not restricted to a niche which would obviously explain their radiation resistance. It may be that their repair capability is vestigial being required in the past to cope with elevated levels of harmful radiation and free radicals present in the primordial environment (Berkner and Marshal 1964). In keeping with this, *D.radiodurans* contains large

quantities of superoxide dismutase which eliminates oxygen radicals, also a feature of the earth's early atmosphere (McCord *et al.*, 1971; Rao and Cammack 1981). More recently, it has been suggested that *Deinococcus spp.* require their repair capacity to survive cosmic-ray bombardment whilst traversing interstellar space!! (Hoyle and Wickramasinghe 1981).

3. Transformation in *D.radiodurans*

Transformation is the uptake of free DNA from the medium by bacteria and the subsequent expression of genes carried on the DNA (Hotchkiss and Marmur 1954). Transformation provides the only known method of genetically analysing *D.radiodurans* to date since the bacteria do not conjugate and there are no known bacteriophages (Moseley 1983). *D.radiodurans*, which is the only transformable *Deinococcus* species, was first demonstrated to be transformable by Moseley and Setlow (1968). Tirgari and Moseley (1980) later improved the efficiency of transformation by adding calcium ions which increased the frequency of transformants to 1% for some markers. *D.radiodurans* is unlike many bacteria which require a competence factor or extreme physiological conditions to effect transformation (Tomasz 1969) and will remain competent throughout exponential growth (Tirgari and Moseley 1980). In fact, measurement of the competent fraction of a population using the unlinked marker technique gives values in excess of 400% providing evidence for multiple transformable units (single DNA strands) within *D.radiodurans* cells (Moseley and Evans (1981).

Transformation provides the only method for distinguishing whether mutant strains of similar phenotype carry mutations in the same or different genes (Moseley and Copland, 1975, 1978) and for establishing gene linkage in *D.radiodurans* (Tirgari 1977).

DNA repair in *D.radiodurans*

The majority of the work on *D.radiodurans* has centred around its resistance to ionizing and UV radiation (reviewed by Moseley 1983). However, the resistance also extends to most DNA damaging agents including β-propiolactone, mitomycin C, ICR191G and bromomethylbenzanthracene (Sweet and Moseley 1974, 1976; Tempest and Moseley 1980).

Early explanations for the resistance of *D.radiodurans* and *D.radiophilus* to UV and γ rays centred upon possible intracellular radioprotective compounds. The carotenoid pigment was shown to have a variable effect on resistance since pigmentless strains have been isolated which were sensitive to these agents whilst others have been isolated which were as resistant as the wild type (Moseley 1963; Okazawa and Matsuyama 1967; Lewis *et al.*, 1974). The high sulphydryl content of the bacterium was also implicated and led to the isolation of a "factor" that was claimed to be responsible for the radioresistance (Bruce 1964). This "factor" could protect *E.coli* from X-irradiation and was postulated to modify the damage to a repairable form (Goldstein *et al.*, 1978). Others have found no correlation between sulphydryl content and radiation resistance (Serianni and Bruce 1968). The high concentration of manganese around

the DNA of *D.radiodurans* was another possible source of radioprotection due to its ability to reduce the yield of UV-photoproducts or modify X-ray damage (Leibowitz *et al.*, 1976). However the measured quantities of UV-photoproducts in the DNA excludes this possibility. Approximately 1% of thymine in DNA is present as thymine-thymine dimers (TT) (see Appendix) at $500Jm^{-2}$ which is a sublethal dose for *D.radiodurans* (Boling and Setlow 1966). Although the above factors may contribute in part to the repair of DNA damage, they must play a minor role since efficient DNA repair mechanisms have been shown to play the major role.

Excision repair of TT dimers was the first DNA repair mechanism to be demonstrated in *D.radiodurans* (Boling and Setlow 1966) and remains the only repair function for which there is direct physical evidence. Pyrimidine dimers are removed as part of short oligonucleotides that are released into the surrounding medium (Boling and Setlow 1966), a situation unlike that in *E.coli* but which parallels other transformable bacteria (Setlow *et al.*, 1968). Cytosine-thymine (CT), thymine-cytosine (TC) dimers and Thy(6,4)pyo adducts (see Appendix) are also excised at the same rate as TT dimers (Moseley 1969; Varghese and Day 1970). Similarly 5,6 dihydroxydihydrothymine, which comprises 5-10% of UV induced (Targovink and Hariharan 1980) or gamma-irradiation induced (Hariharan and Cerutti 1971) damage in the DNA, is also excised. The DNA is incised in response to UV damage (Moseley 1969) and the subsequent excision repair operates in the absence of protein synthesis (Boling and Setlow 1966) but further details of the nature of the excision process, including the enzymes involved, remains unknown.

The presence of a second repair mechanism based on recombinational exchanges in DNA has been inferred from several lines of evidence but has not been physically demonstrated. Such evidence includes the ability of *D.radiodurans* to repair a large number of double strand breaks (260 per genome) (Dean *et al.*, 1966; Kitayama and Matsuyama 1968, 1971). One or two double strand breaks can be repaired in *E.coli* which requires recombinational events and a homologous chromosome (Krasin and Hutchinson 1977). The requirement for homologous chromosomes in *D.radiodurans* is fulfilled by the presence of multiple genomes in each nucleus (Hansen 1978; Tirgari and Moseley 1980; Moseley and Evans 1981). The multiple genomes may provide a recombinational reservoir of undamaged DNA templates (Moseley and Evans 1981). However it has since been demonstrated that UV-resistance does not correlate well with the number of genomes present (Harsojo *et al.*, 1981). Also, several UV-sensitive bacteria have been found to possess a large number of genomes in the nucleus (Sadoff *et al.*, 1979; Purvis pers. comm.). The possibility remains though that additional chromosomes are available for recombination in *D.radiodurans*.

Recombination-type repair has also been inferred from the slow rejoining of gamma ray-induced DNA single-strand breaks in *D.radiodurans* which appears similar to "Type III" recombination repair in *E.coli* in requiring protein synthesis (Dean *et al.*, 1970; Hariharan and Cerutti 1972; Town *et al.*, 1973). The shoulder of the *D.radiodurans* UV survival curve has previously been attributed to recombinational exchanges on the basis of the loss both of transformability and the

shoulder when temperature-sensitive DNA synthesis mutants are shifted to the restricted temperature (Moseley *et al.*, 1972). A UV sensitive mutant of *D.radiodurans*, rec 30, has also been isolated which appears to be recombination repair defective on the basis of its loss of transformability and its extreme sensitivity to DNA crosslinking agents and X-rays suggesting that it may be similar to a *recA* strain of *E.coli*, although it differs from a *recA* strain in that it does not display 'reckless' DNA degradation following damage (Moseley and Copland 1975).

As well as being resistant to the lethal effects of DNA-damaging agents relative to other organisms *D.radiodurans* is also immutable by all except those which directly cause mispairing in replication (Sweet and Moseley 1974, 1976). This appears characteristic of *Deinococcus spp.* (Tempest and Moseley 1982) leading to the conclusion that error-prone repair mechanisms are absent from the group.

D.radiodurans also appears to lack an adaptive repair response towards O^6 methylguanine (Tempest 1978), although an adaptive repair of mitomycin C crosslinks has been reported on the basis that their removal is prevented in the absence of protein synthesis, but not if cells are preinduced by growth in low doses of mitomycin C (Kitayama 1982). Protein synthesis is also known to be required for the repair of X-ray damage (Dean *et al.*, 1970; Kitayama and Matsuyama 1971; Kitayama *et al.*, 1981; Driedger 1971) and four proteins have been identified as being induced by DNA damage (Hansen 1980). However, specific proteins have not been correlated with particular repair phenomena.

Very few enzymes have been identified in *D.radiodurans* and none of these has been directly implicated in DNA repair. The only possible exception is a DNA polymerase identified by Gentner (1973, 1974) which is required for repair of some X-ray damage, the quantity of polymerase increasing after X irradiation. EDTA has also been shown to inhibit resealing of gaps produced after X-ray damage (Driedger 1971) which may be due to the inhibition of 'DNA polymerase 1' identified by Kitayama and Matsuyama (1977, 1978). This has properties in common with *E.coli* DNA polymerase 1, including its dependence on Mg^{2+} ions.

DNA repair-deficient strains of *D.radiodurans*

Several strains of *D.radiodurans* have been isolated by mutagenesis of the wild type strain that are sensitive to UV radiation, gamma rays or mitomycin C (Moseley 1967, 1969; Moseley and Copland 1975, 1978; Okazawa and Matsuyama 1967; Suhadi *et al.*, 1971, 1972; Kitayama 1975). However, the nature of the repair defects in the mutant strains is generally not known. Those mutant strains about which most is known are described below.

Strains UV17 and UV38 were isolated from *D.radiodurans* R1 as being UV sensitive but were found in addition to be sensitive to gamma rays, mitomycin C and MNNG (Moseley 1967). Both strains are apparently normal with respect to excision repair of thymine-thymine dimers and are incision proficient but appear to excise cytosine-thymine dimers at a rate slower than the wild type, although this may have been a failure to remove Thy(6,4)pyo cytosine-thymine adducts which cochromatograph

with cytosine-thymine and thymine-cytosine pyrimidine dimers in the assay system used (Moseley 1969; Patrick and Rahn 1976). Further work on these strains is confused since Gentner (1973) found that polymerase activity was only 5 to 20% of that of the wild type and nuclease activity was normal in both strains whereas Bonura and Bruce (1974) found that polymerase activity was normal in UV17 and nuclease activity was enhanced. Both strains however, appear to be defective in a post-incision step of excision repair in that the rejoining of UV-induced (Moseley 1969) and X-ray induced (Bonura and Bruce 1974) single DNA strands is slow.

An unusual feature of the killing of *D.radiodurans* by UV is that the bacteria are equally sensitive to 254nm UV or 280nm radiation (Setlow and Boling 1965) which led to the suggestion that other kinds of damage to DNA and to protein may ultimately kill the cell and not pyrimidine dimers which are efficiently removed (Setlow and Boling 1965; Jagger 1976; Smith 1976). Strains UV17 and UV38 however have reverted to the expected action spectrum i.e. that of *E.coli* which is more sensitive to 254nm than 280nm radiation (Moseley 1969). The repair of DNA damage induced at 254nm i.e. pyrimidine dimers is therefore defective in these strains.

Strain rec.30, as mentioned earlier, is presumed to be deficient in a recombination function because of its lack of transformability and its sensitivity to mitomycin C (300 fold) and X-rays (100 fold) relative to the wild type (Moseley and Copland 1975). Acute sensitivity to these agents may be expected of a recombination-deficient strain by extrapolating from *E.coli* (Cole 1973). The lack of excessive DNA degradation in this

strain which would occur in a *recA* *E.coli* strain has been explained by a suggestion that *D.radiodurans* lacks a rec BC type exonuclease. (Moseley and Copland 1975).

Four repair-deficient strains were isolated by Moseley and Copland (1978) as being sensitive to UV, in the case of strains 303 and 263, and mitomycin C, in the case of strains 302 and 262. The mutations in these strains are apparently in different genes as determined from restoration of the wild type phenotype at appropriate frequencies in reciprocal transformations between strains. The genes were called *uvsA*, *uvsB*, *mtcA* and *mtcB* respectively. Mutation in genes *uvsA* and *uvsB* produces a UV-sensitive, γ-ray-sensitive, mitocycin C-sensitive phenotype, whereas mutation of *mtcA* or *mtcB* produces only sensitivity to mitomycin C. Data obtained on the relative sensitivities of strains mutant in these genes suggested that the *uvsA* and *uvsB* genes act before the *mtcA* and *mtcB* genes in repair of mitcmycin C crosslinks. The number of genes controlling sensitivity to mitomycin C has recently been extended to eight (Kitayama *et al.*, 1983).

The nature of the defects in strains 303 and 263 have not been further investigated, however, work on strain 302 reveals that the *mtcA* gene is essential for repair of the lethal damage induced by a wide range of alkylating agents and also the premutagenic lesions induced by agents such as MNNG. Strain 302 is therefore strongly mutated by MNNG (and other directly acting mutagens) which is apparently due to a failure to excise O^6 methylguanine from DNA. Strain 302 also cannot incise DNA damaged with bromomethylbenzanthracene which suggests that the *mtcA* gene codes for an endonuclease or a

glycosylase which has a wide spectrum of substrates (Tempest and Moseley 1980).

Scope of the present work

The aim of this thesis is to characterise the excision pathway for the repair of DNA damage (particularly UV damage) in *D.radiodurans*. No repair-deficient strain of *D.radiodurans* has been isolated previously that has been shown to be defective in the repair of the commonest UV photoproduct namely pyrimidine dimers. Strains of *D.radiodurans* which have the putative phenotype of excision repair deficient strains of *E.coli* were therefore isolated as part of this project and examined along with previously-isolated strains in an effort to identify truly excision repair deficient strains for the first time. Different parameters associated with excision repair of DNA damage were examined in these strains to establish at what point in the excision repair process particular mutations produce their effect and also whether a particular repair deficient phenotype was the result of an excision-repair defect. The number of genes controlling excision repair was also investigated and the particular effect that each gene has on excision repair. The consequences of the loss of excision repair were examined with a view to assessing the contribution that particular excision repair mechanisms make to the overall repair capacity of the organism.

Several attempts have been made in the past to identify enzymes in *D.radiodurans* which can be related to a particular DNA repair process, including attempts to identify activities which specifically incise UV-irradiated DNA (T. Bonura and

C.A. van Sluis pers.comm.). However, these efforts were in vain partly due to the preponderence of contaminating non-specific nucleases in *D.radiodurans*. An effort was made to overcome the non-specific enzymes and to subsequently identify DNA-damage specific DNA incising activities in cell free extracts, especially activities towards DNA containing pyrimidine dimers. Characterisation of these activities enabled comparisons to be made with the well-characterised UV incision enzymes of *E.coli*, T4 and *M.luteus*. The genetic control of these enzymes was also studied in appropriate repair-deficient strains of *D.radiodurans*.

Some of this work has been reported elsewhere (Moseley and Evans 1983; Evans and Moseley 1983).

CHAPTER 1

Section 2: DNA repair in other organisms.

This section is intended to give an overall picture of DNA repair to put the work done previously on *D.radiodurans*, and that reported in this thesis, into perspective.

1. DNA repair - General

Lesions in DNA are removed by a variety of mechanisms that restore the integrity of the DNA and thereby its genetic function. A useful division in these mechanisms is between the predominant 'error free' enzymatic pathways which lead to accurate restoration of genetic function and the 'error prone' mechanisms which repair DNA structure at the expense of the original nucleotide sequence and lead to mutation. Alternatively DNA repair can be divided according to whether DNA damage is (i) removed directly without interruption of the DNA overall structure, (ii) removed *via* the sequential breakdown and resynthesis of the damaged region of DNA, or (iii) tolerated until it is diluted by successive cell divisions or it can be removed by (i) or (ii). The types of repair which operate in (iii) rely on post-replication recombinational exchanges and are not "true" repair since damage persists in the DNA. The second form of repair, excision repair, is most relevant to this thesis and is discussed in most detail in Section 3.

2. Direct removal of DNA damage

Photoreactivation. Photoreactivating enzymes catalyse the light dependent (300-600nm) chemical reversal of the cyclobutane ring of pyrimidine dimers thereby restoring the integrity of adjacent pyrimidines (reviewed by Sutherland 1981). The reaction takes place in two steps, firstly the enzyme binds to a pyrimidine dimer in the dark or light to form a stable complex. Upon exposure to light the complex then absorbs a photon of light which provides the energy to cleave the pyrimidine dimer into two pyrimidines. *D.radiodurans* is relatively unusual in lacking this form of repair (Moseley 1983).

Alkyl transferases and the adaptive response. When *E.coli* is exposed to low doses of simple alkylating agents such as MNNG it acquires resistance to the lethal and mutagenic effects of a subsequent, higher dose (Samson and Cairns 1977). The acquisition of this resistance is called "the adaptive response" (Jeggo 1977) and is caused by an increased capacity to repair various alkylated DNA bases as a consequence of the induction of two different enzymes. The enzyme responsible for resistance to mutation is an alkyltransferase that transfers alkyl groups from O^6 alkyl guanines (which are mutagenic) to a cysteine residue in the enzyme in a suicidal reaction which inactivates the enzyme and restores the guanine (Schendel and Robbins 1978; Karran *et al.*, 1979; Olsson and Lindahl 1980; Foote *et al.*, 1980). The second enzyme is 3-methyl adenine glycosylase II which initiates base excision repair of 7 alkyl guanine, 3 alkyl adenine and 3 alkyl guanine, the

last two of which are lethal lesions (Lawley and Thatcher 1970; Evenson and Seeberg 1982; Karran *et al.*, 1982). Alkyl transferases and 'adaptive responses' have been identified in many other organisms such as rodents (Lemaitre *et al.*, 1982), humans (Lindahl *et al.*, 1982), *M.luteus* (Riazuddin *et al.*, 1983) and *B.subtilis* (Mitra *et al.*, 1983) but appear to be absent from *D.radiodurans* (Tempest 1978).

3. Excision repair of DNA damage in general

Excision repair proceeds by a sequence of enzymatic steps that results in the physical removal of damage from DNA. It was discovered when Boyce and Howard Flanders (1964) and Setlow and Carrier (1964) observed the transfer of thymine-thymine dimers from the trichloroacetic acid (TCA)-insoluble to the TCA-soluble fraction of wild type *E.coli* but not in the UV sensitive B_{s-1} strain or if a mutation was present in *uvrA*. Subsequent work produced a model of the excision repair process that remained largely unchallenged until recently. This 'classical' scheme of events was divided into four stages (i) a single strand scission of a phosphodiester bond in DNA occurs near to and in the same strand as a lesion, (ii) exo-nucleases degrade from the scission removing the single strand region containing the lesion, (iii) DNA polymerase 1 resynthesises the missing DNA using the intact strand as a template, (iv) the newly synthesised DNA is linked covalently to the existing DNA by DNA ligase. This model has since been modified with respect to the repair of some forms of damage and variations appear to exist between organisms, all of which is described in detail in Section 3.

4. Tolerance of DNA damage by postreplication, recombination repair

The existence of a form of repair which depends on recombinational exchanges was first suggested by Howard-Flanders and Boyce (1964) and was later supported by the observation that recombination deficient (*recA*) strains of *E.coli* were UV sensitive (Clark and Margulies 1965), but excision proficient (Clark *et al.*, 1966). Also, it was found that a *recA uvrB* double mutant was more UV sensitive than strains carrying either single mutation which suggested that the *recA* dependent repair pathway was distinct from the *uvrA* dependent pathway. In *uvrA* strains pyrimidine dimers remain in the DNA (Howard-Flanders *et al.*, 1966b) and DNA synthesis proceeds slowly, with the molecular weight of the DNA being inversely proportional to the UV dose and approximating to the interdimer distance (Rupp and Howard-Flanders 1968; Sedgwick 1975; Youngs and Smith 1976). Only the undamaged regions of DNA are replicated with each new chain terminating at a dimer (Rupp and Howard-Flanders 1968) (see fig.2A and B). The replication complex then resumes synthesis approximately 1000 nucleotides further along the template strand which leaves a daughter strand gap (fig.2B) (Iyer and Rupp 1971) which is not a substrate for excision repair (Howard-Flanders *et al.*, 1968). The new DNA strands remain low molecular weight for about 15 minutes and then become part of longer DNA strands (Rupp and Howard-Flanders 1968; Rupp *et al.*, 1971; Howard-Flanders *et al.*, 1968). Pyrimidine dimers appear in newly synthesised DNA as a result of the exchanges and become diluted as they are distributed between new and old DNA strands (Ganesan 1974; Ganesan and

Figure 2. Current model of the exchanges in DNA associated with postreplication, recombination repair.

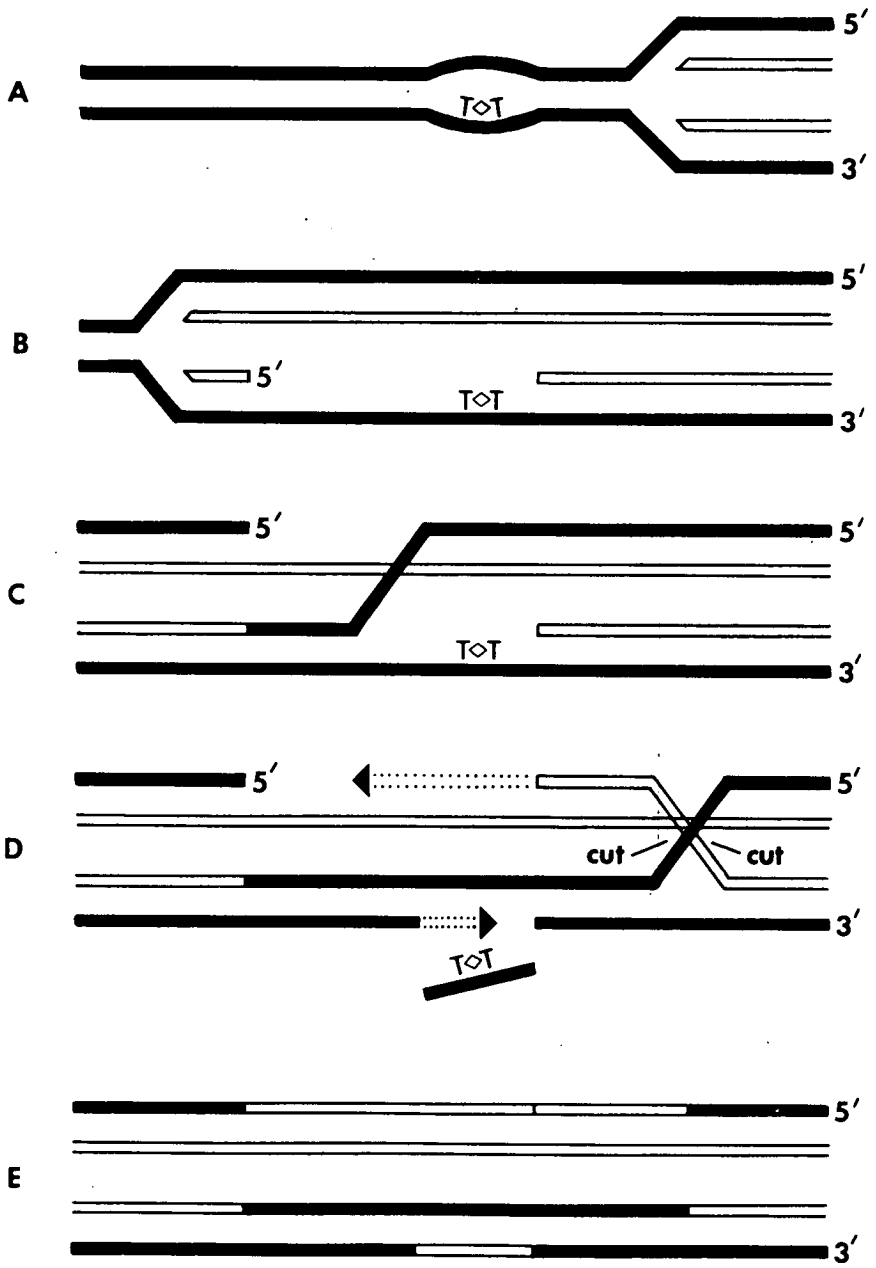
A - The replication fork approaches a pyrimidine dimer.

B - A daughter strand gap is created due to a failure to replicate a pyrimidine dimer.

C - A break is introduced in a parental DNA strand. The 3'-OH end is assimilated into the daughter strand gap by the *recA* protein.

D - The *recA* protein drives the strand transfer over the dimer and causes reciprocal strand transfer *via* a Holliday structure. The pyrimidine dimers can then be repaired *via* excision repair.

E - Repaired DNA.
Newly synthesised DNA is in white and parental DNA is in black.


A
5′
TOT
3′
B
5′
5′
TOT
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C
5′
5′
TOT
3′
D
5′
5′
cut
cut
3′
TOT
E
5′
3′

Seawell 1975). The process is absent from *recA*⁻ strains (Smith and Meun 1970). The size of the exchanged region has been measured by a variety of methods at between 3,000-40,000 nucleotides (refs. in Hanawalt *et al.*, 1979).

The postulated mechanism of the recombinational exchanges requires there to be an homologous DNA duplex in the vicinity of the daughter strand gap. Exchange of DNA single strands then occurs *via* a sequence of steps involving breakage of DNA single strands, heteroduplex formation, strand exchange and religation of DNA strands (Radding 1978). The *recA* protein has been directly implicated in this process *in vivo* (Holliman *et al.*, 1975; Holliman and Radding 1976) and *in vitro* where it has been shown to promote homologous pairing of single strands to duplex DNA (McEntee *et al.*, 1979; Shibata *et al.*, 1979), the pairing of duplex DNA to daughter strand gaps (Cassuto *et al.*, 1980; Cunningham *et al.*, 1980), the transfer of DNA single strand to form a heteroduplex (DasGupta *et al.*, 1980; West *et al.*, 1981a, 1981b, Cox and Lehman 1981) and the pairing of two DNA duplexes into a four strand heteroduplex (DasGupta *et al.*, 1981). These reactions have been combined *in vitro* into the complete reciprocal exchange of single strands between two DNA duplexes by transferring the 3'-OH end of a nicked duplex into a single strand gap (see fig.2C), the *recA* protein then drives the heteroduplex along the DNA which completes the exchange (West *et al.*, 1981b, 1982). The reaction will also occur with UV-irradiated DNA, the heteroduplex formation continuing over pyrimidine dimers and completing the reciprocal exchange (see fig.2D). The dimers can then be repaired by excision repair (Livneh and Lehman

1982) (fig.2D).

Mutations in genes other than *recA* affect the ability of *E.coli* to perform postreplication repair *in vivo*; these include *lexA* (Youngs and Smith 1976; Sedgwick 1976), *recB*, *recC*, *recF* (Clark 1973; Ganesan and Seawell 1975; Youngs and Smith 1976; Lloyd and Thomas 1983), *uvrD* (Youngs and Smith 1976), *dnaG* (Johnson 1976), *polB* and *polC (dnaE)* (Tait *et al.*, 1974; Sedgwick and Bridges 1974; Johnson 1978) and possibly *uvrA* and *uvrB* (Hanawalt *et al.*, 1979), although the details of the involvement of most of these genes is not well understood. Evidence obtained for the involvement of the UVRABC enzyme in the original strand breakages is contradictory; in some instances postreplication repair appears dependent on the *uvr* genes under SOS-inducing conditions (see next section) (Mount *et al.*,1976; Rothman *et al.*, 1979; Castellazzi *et al.*, 1980) whereas other evidence suggests the opposite (Radman and Devoret 1971; Witkin 1976; Boyle and Setlow 1970).

The recombination repair thought to exist in *D.radiodurans* is likely to be similarly complex and some of the strains of *D.radiodurans* described in this thesis may be defective in some aspect of a recombination repair mechanism.

5. Regulation of DNA repair

The exposure of exponentially-growing *E.coli* to DNA-damaging agents such as UV radiation or mitomycin C results in the induction of a wide range of cell responses collectively referred to as the "SOS" response (Radman 1975; Witkin 1976; 1982; Little and Mount 1982). The responses affecting DNA repair include enhanced excision repair (Kenyon and Walker

1981; Sancar *et al.*, 1982a), postreplication repair (Smith *et al.*, 1978), enhanced radiation resistance (Pollard *et al.*, 1981), inhibition of DNA degradation by exonuclease V (Pollard and Randall 1973) and mutagenesis *via* error-prone DNA repair (Kato and Shinara 1977; Shanabruch and Walker 1980; Kenyon and Walker 1981). The central feature of control of the SOS response is the *lexA* protein which is a repressor of its own synthesis and the synthesis of nine or more other gene products including that of the *recA* gene (Brent 1982). Apart from participating in some repair events directly (Livneh and Lehman 1982) the *recA* protein interacts with the *lexA* protein in a regulatory control circit, the current model of which (see Fig.3) involves an 'off' state where DNA is not damaged and the *lexA* protein binds to the operator sites of the genes it represses (Brent 1982). Pathways of repair that depend directly on the *recA* protein and other proteins involved in DNA repair therefore are either not operative or operate at reduced levels. When DNA is damaged a signal, possibly the production of short oligonucleotides or single stranded DNA or the appearance of single strand gaps, activates the *recA* protein which becomes a protease. The protease cleaves the *lexA* protein thereby allowing synthesis of the gene products under its control (which includes its own synthesis). In this 'on' state, the SOS responses that depend on these gene products are manifested. Intermediate states between 'on' and 'off' are achieved by intermediate quantities of DNA damage producing a weaker inducing signal (Little and Mount 1982; Brent 1982; Witkin 1982). *recA* dependent DNA repair pathways were originally thought to be

Figure 3: Summary of the SOS regulatory circuit for the control of genes whose synthesis is repressed by the *lexA* protein.

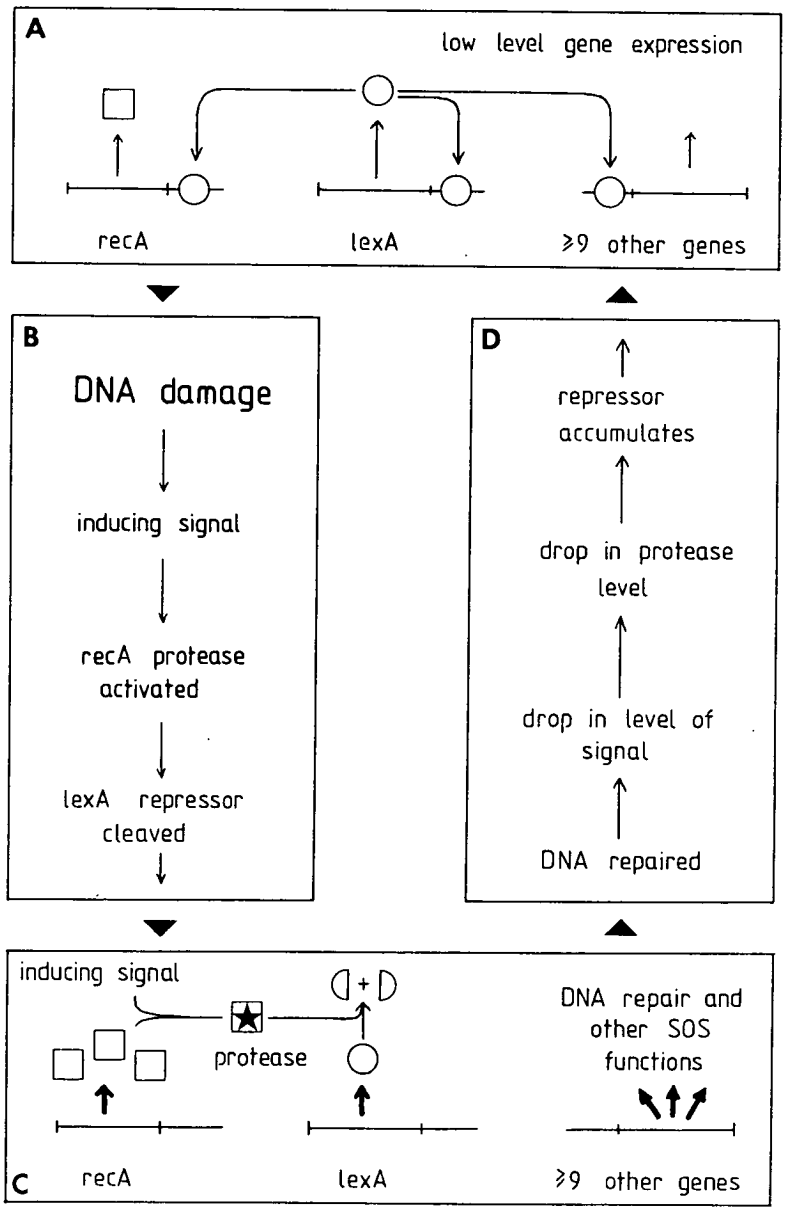
A - Normal low levels of expression of *lexA* repressed genes (including *lexA*) in undamaged cells.

B - Induction of the SOS response to DNA damage.

C - Derepressed synthesis from the genes under *lexA* control.

D - Restoration of low levels of expression of genes under *lexA* control as DNA repair is completed.

Taken from Little and Mount (1982).


A
low level gene expression
recA
lexA
≥9 other genes
B
DNA damage
inducing signal
recA protease activated
lexA repressor cleaved
C
inducing signal
protease
recA
lexA
≥9 other genes
DNA repair and other SOS functions
D
repressor accumulates
drop in protease level
drop in level of signal
DNA repaired

distinct from excision repair (Hanawalt *et al.*,1979). However, the *uvrA* (Kenyon and Walker 1981), *uvrB* (Fogliano and Schendel 1981) and *uvrD* (Siegel 1983) genes have been shown to be SOS inducible *via* operon fusions with Mud (Ap*lac*) phage (Casadaban and Cohen 1979). The operators of these genes contain promoters which are directly repressed by *lexA* protein which binds to a common (similar) nucleotide sequence which lies within the promoter regions. This consensus sequence is also found within the *recA* and *lexA* operators and is termed an "SOS box" (Little and Mount 1982; Sancar *et al.*, 1982a; Kushner *et al.*, 1983). The *uvrB* gene contains three promoters termed (starting from that closest to the structural gene) P_1, P_2 and P_3. *lexA* protein binds to P_2 and also covers part of P_3 and possibly P_1. Since P_1 and P_3 could conceivably be directly repressed by repressors other than *lexA*, overall control of the structural gene may be extremely complex (Sancar *et al.*, 1982; Van der Berg *et al.*, 1983). . The *uvrC* operator also contains three promoters which are under an unidentified form of control. A protein synthesised from the region between the most distal promoter and the structural gene may also have a role in control of *uvrC* expression (Sharma and Moses 1983). The *uvrD* gene also has three promoters, one of which is not under direct *lexA* control (Kushner *et al.*, 1983). There are therefore a variety of routes for control of the complete UVRABC enzyme and hence excision repair and also other repair pathways which depend upon the *uvrA*, *uvrB*, *uvrC* and *uvrD* genes (see Fig.4). *lexA* repression of *uvrA* and *uvrB* is surprising at first sight since excision repair is operative in *recA*$^-$ strains (Clark and Volkert 1978) and in the absence

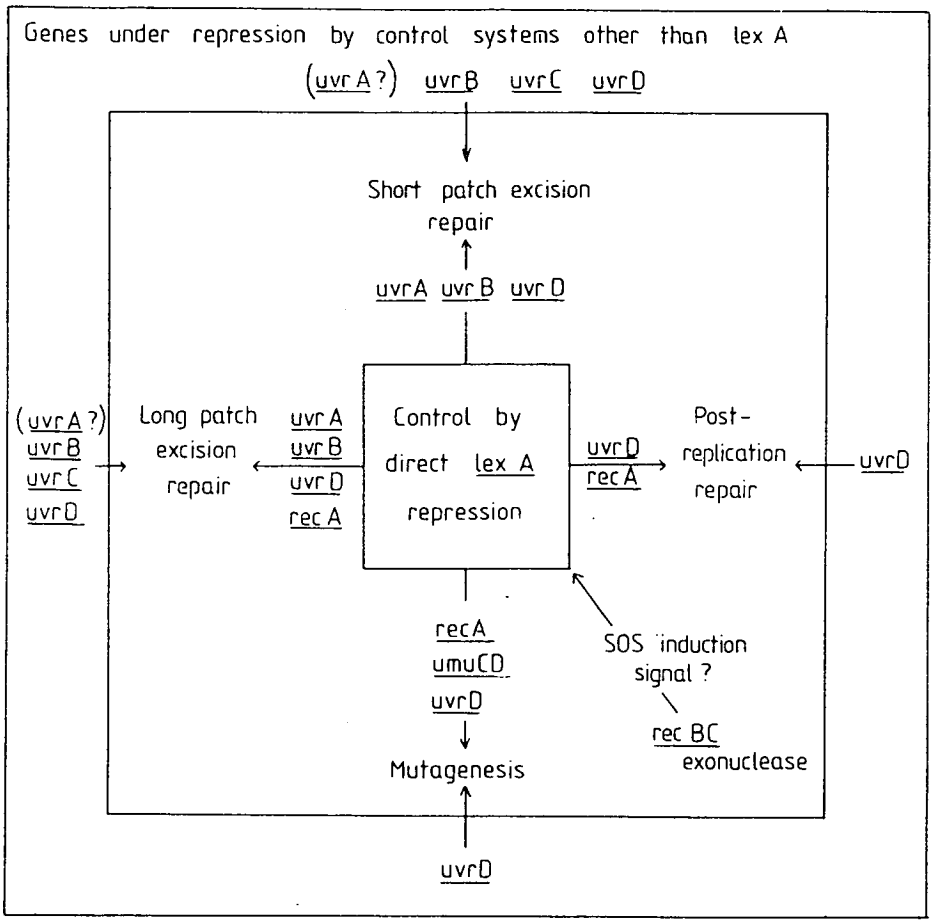


Figure 4

Summary of possible routes for the control of DNA repair mechanisms in *E.coli via* genes associated with excision repair.

of protein synthesis (Boyle and Setlow 1970). This indicates that the basal levels of excision enzymes (ie under non-SOS inducing conditions) are large enough to cope with most DNA damage in *E.coli*. However, the SOS dependence of these enzymes is probably the source of the enhanced radiation resistance of SOS induced *E.coli* (Pollard *et al.*, 1981). The contribution made by promoters other than those repressed by *lexA* is not known but the observed 1:7 ratio of UVRA to UVRB in *recA* *E.coli* cells (Sancar *et al.*, 1981) may be a reflection of enhanced synthesis of UVRB *via* the P_1 promoter (Sancar *et al.*, 1982a).

Several proteins are synthesised in response to DNA damage in *D.radiodurans* (see earlier) suggesting the presence of some form of 'SOS'-like response. A component of this response will be shown to have a profound effect upon one aspect of excision repair in *D.radiodurans*.

6. DNA repair and mutagenesis

Mutations arise *via* two main routes, firstly as replication errors introduced either spontaneously or by chemical modification of DNA nucleotides, and secondly due to misincorporation of nucleotides during DNA repair. In the first case DNA repair mechanisms are employed to remove the mutagenic lesions and the emphasis is for repair mechanisms to minimise mutation, whereas in the latter case the toleration of the damage is at the expense of introducing mutations. Particular DNA repair mechanisms were found to contribute differently to mutation by the observation that UV induced mutation is eliminated in *recA*$^-$ or *lexA*$^-$ *E.coli* strains (Kondo 1973; Witkin and Parisi

1974). Repair independent of *recA* and *lexA* was therefore dubbed 'error-free', ie short patch excision repair and direct removal of damage, whereas the *recA lexA*-dependent repair was 'error prone' (Witkin 1969; Bridges 1969). *Uvr*$^-$ strains were also found to be hypermutable which was assumed to be due to the majority of lesions being repaired by *recA lexA* error-prone repair (Witkin 1969). The mutation was not *via* recombination however since mutation occurred in the absence of both the *recBC* and *recF* recombination pathways (Witkin 1969). However, in a *uvrD*$^-$ background a *recBC*$^+$ phenotype is required for mutation (Smith *et al.*, 1978). Both the *uvrD* and *recBC* pathways also depend on a functional *umu* operon for mutation production (Kato 1977; Kato and Shinoura 1977). The function of the *umu* operon, which contains two genes, *umuC* and *umuD* (Shinagawa *et al.*, 1983), is unknown but it has been suggested that it may modify a polymerase (ie. is a protein kinase or methylase) making it error-prone (Villani *et al.*, 1978). The *umu* operon is also similar to a class of plasmid born transposon-like genes (muc) which enhance mutogenesis and some of which at least can be *lexA* repressed (Bagg *et al.*, 1981; Langer *et al.*, 1981; Walker *et al.*, 1982). The existence of bacteria that are immutable by UV, such as *Proteus spp.* (Böhme 1963). *Haemophilus spp.* (Kimball *et al.*, 1977) and *Deinococcus spp.* (Sweet and Moseley 1976; Tempest and Moseley 1982) has led to the suggestion that these bacteria lack a *umu*-like function (Walker *et al.*, 1982). Indeed, introduction of the pKM101 *muc* genes into *Proteus mirabilis* renders the cells UV mutable (Hofemeister *et al.*, 1979).

UV-induced mutation appears to have two components, 'untargeted' mutagenesis where mutations are not at the sites of pyrimidine dimers and can even occur in the absence of damage in a *tif E.coli* strain (Witkin and Wermundsen 1979) and 'targeted' mutagenesis which is probably due to replication across a pyrimidine dimer (Caillet-Fauquet *et al.*, 1977). Untargeted mutagenesis may be due to a relaxation of the fidelity of DNA polymerase I (Lackey *et al.*, 1982) and/or DNA polymerase III (Bridges and Mottershead 1976; Echols 1982), although *polA* mutations have little effect on mutagenesis (Kondo *et al.*,1970; Witkin and George 1973). Targeted mutagenesis may occur due to the *recA* protein coating the single-stranded region of the lesion which allows replication at the expense of fidelity (Echols 1982). It has always been assumed that pyrimidine dimers are the mutagenic lesion after UV irradiation because firstly they are the predominant lesion, secondly photoreactivation abolishes UV mutation and is specific for monomerization of pyrimidine dimers (Sutherland 1981), and thirdly excision repair-defective mutants show increased sensitivity to the lethal and mutagenic effects of UV light (Witkin 1969). However, examination of UV-mutated DNA at the nucleotide level in phage M13 (Schaaper and Glickman 1982), plasmids (Livneh 1983) and the *lacI* gene (Brash and Haseltine 1983) reveal that mutations which are 'untargeted' with respect to pyrimidine dimers are well correlated with TC and CC sequences (Lippke *et al.*, 1981; Brash and Haseltine 1982) which are the sites of the minor (10% of TT dimers) Thy (6-4) pyo photoproducts (see appendix A, Haseltine 1983; Patrick and Rahn 1976). Thy (6-4) pyo photoproducts are

excised from DNA by the UVRABC enzyme (Franklin *et al.*, 1982; Sancar and Rupp 1983) which would be consistent with an increase in mutation in *uvr*$^-$ strains. The reduction in mutation previously observed after photoreactivation could be explained if the pyrimidine dimers were primary SOS response inducers rather than primary mutagenic lesions (Haseltine 1983). The proportion of thy(6-4)pyo photoproducts to cyclobutane pyrimidine dimers is approximately 1:6 in *D.radiodurans*, and approximately 1200 can be tolerated per genome without mutation. Untargeted and targeted UV mutagenesis mechanisms are both absent from *D.radiodurans*.

Spontaneous mutations can arise due to both rare tautomeric forms of bases occurring during replication (Watson and Crick 1953) and the fidelity of the replication machinery being finite. The fact that DNA polymerase activity of bacterial polymerases is of lower fidelity in isolation *in vitro* than the observed spontaneous mutation frequencies *in vivo* suggests that correction of misincorporated bases occurs (Kornberg 1981). Two mechanisms have been identified that are capable of achieving this. Firstly the 3'→5' exonucleases of the DNA polymerases in various organisms, including *E.coli* and phage T4, will remove misincorporated nucleotides immediately after they are inserted and mutations which increase or decrease these activities cause corresponding antimutator or mutator phenotypes respectively (Kornberg 1981; Villani *et al.*, 1978; Echols 1982). However, if one or more nucleotides are added to a mispaired nucleotide then the exonucleases are probably unable to 'proofread' any longer. The misincorporated nucleotide may however distort the DNA when mispaired and this distortion is probably the substrate

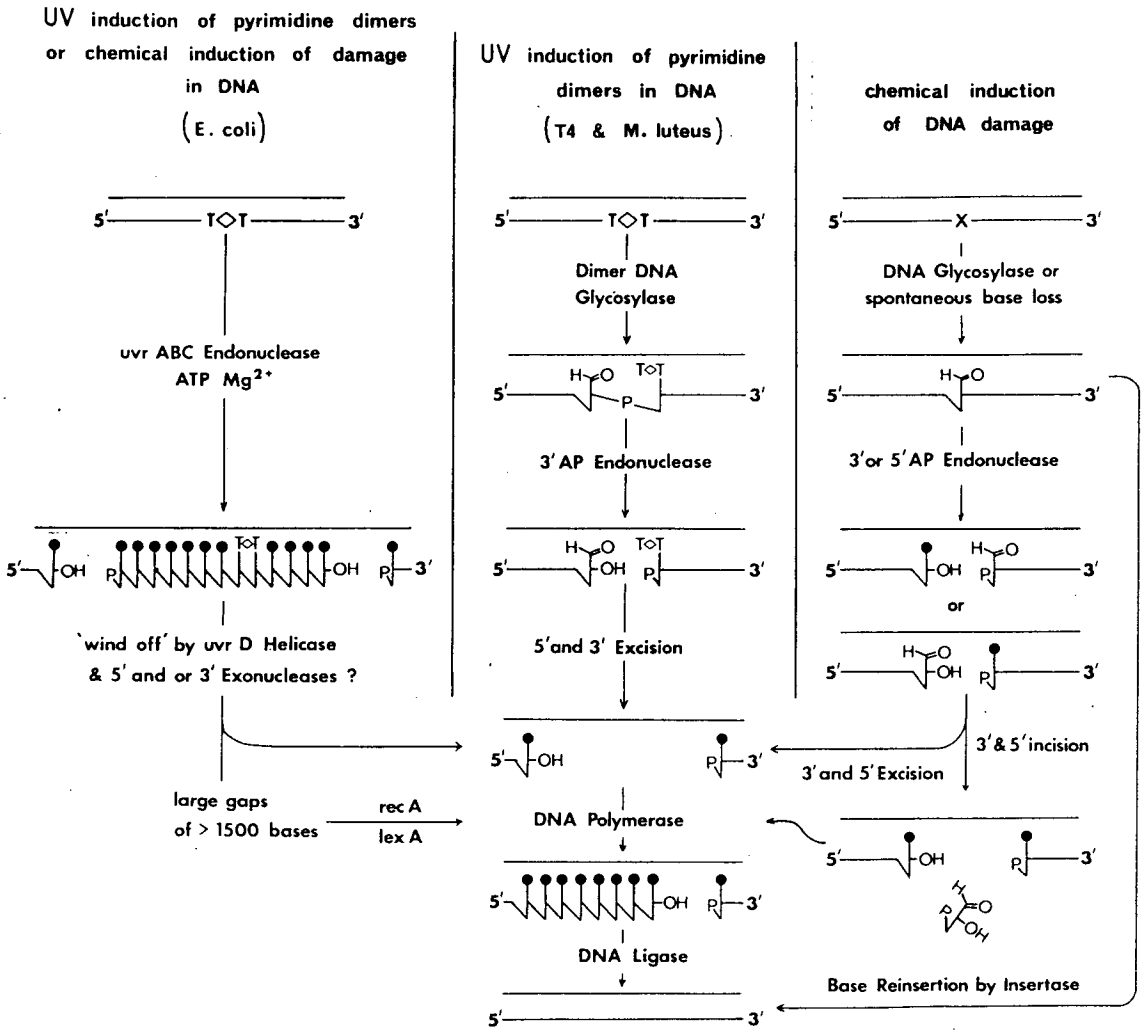
for the second method of correcting replication errors called 'mismatch repair' which excises incorrect nucleotides (Radman 1978; Caillet-Fauquet and Maenhaut-Michael 1982; Maenhaut-Michael and Caillet-Fauquet 1982). An essential feature of this repair is the undermethylation of newly synthesised DNA which allows discrimination between the "correct" parental DNA strand and the "incorrect" mispaired base of the daughter strand (Radman *et al.*, 1980). DNA is generally methylated as part of the restriction and modification systems of bacteria (Marinus and Morris 1973; Lacks and Greenberg 1977). However, a lag probably occurs between replication and methylation of daughter strands which provides what is presumably a short period in which mismatch repair can operate (Radman *et al.*, 1980). A consequence of a failure to methylate DNA in *dam*$^-$ mutants is a high spontaneous mutation rate due to a failure to discriminate between parental and daughter DNA strands (Marinus and Morris 1975) and extreme sensitivity to the lethal effect of base analogues (Glickman *et al.*, 1978). Excision *via* mismatch repair depends on the *mutU*, *mutH*, *mutS* and *mutL* genes (Radman *et al.*, 1980) which act independently of excision repair and error-prone post-replication repair (Maenhaut-Michael and Caillet-Fauquet 1982). Mismatch repair seems not to be involved in the damage removal directly (Maenhaut-Michael and Caillet-Fauquet 1982) but rather can repair UV induced, error prone repair-generated, mismatches (Caillet-Fauquet and Maenhaut-Michael 1982). Mismatch repair also occurs in response to MNNG alkylation due to the recognition and removal of alkylated bases such as O^6 methylguanine which are not mispaired (Jones and Wagner 1981; Karran and

Marinus 1982). *D.radiodurans* appears to lack methylase activity and methylated DNA bases and so mismatch repair of the *E.coli* type may be absent. However, some form of mismatch repair may operate using the resident form of modification, the form of which is unknown (see earlier).

CHAPTER I

Section 3: Excision repair of DNA damage

Figure 5 Summary of DNA excision repair pathways



Incision mechanisms

Until recently the incision of damaged DNA was in all cases considered to be due to a single-step process achieved by a range of endonucleases which characteristically recognise particular types of DNA damage (Grossman *et al.*, 1975), the model system for this being the production of incisions in response to pyrimidine dimers by the multi component 'UV endonuclease' of *E.coli* (Hanawalt *et al.*, 1979). However, in many cases the endonuclease activity in response to several forms of chemically-induced damage was found to consist of the combined action of a glycosylase and an apurinic endonuclease, the former conferring the damage specificity (Lindahl 1976). Later still the apparent UV endonuclease activities of *M.luteus* and bacterophage T4, which were assumed to be similar to the *E.coli* UV endonuclease, were found to consist of the combined action of a glycosylase and an apyrimidinic endonuclease (Demple and Linn 1980). This led to a division in the classification of excision repair. When breakage of a DNA phosphodiester bond is preceded by glycosylase action, the subsequent repair is often termed "base excision repair" and when the breakage is a one step process involving only endonuclease action the subsequent repair is referred to as "nucleotide excision repair" (Lindahl 1976, 1979; Duncan *et al.*, 1976). The recent discovery that the *E.coli* UV endonuclease produces two incisions for each DNA lesion (Sancar and Rupp 1983) means that there are three types of incision. (1) Two incisions produced by one enzyme, (2) one incision produced by one enzyme and (3) one incision produced by the cooperative effect of two enzymes.

The second type which was originally thought to describe all DNA repair incisions now has few, if any, representatives. In this thesis type (1) incisions and type (2) are considered as nucleotide incision repair.

(a) Nucleotide Excision Repair

Damage-specific incision of the type which predominates in *E.coli* for the repair of pyrimidine dimers requires several gene products and has only been identified unequivocally in *E.coli*, although the predominant excision repair mechanism in yeast (Wilcox and Prakash 1981) and mammalian cells (Hanawalt *et al.*, 1979) is thought to proceed *via* a comparable mechanism.

Nucleotide excision repair in *E.coli* is governed by the *uvrA*, *uvrB* and *uvrC* genes and mutations in any one of these renders the cell sensitive to a wide variety of DNA-damaging agents including UV, nitrous acid (Howard-Flanders and Boyce 1966), mitomycin C (Boyce and Howard-Flanders 1964) and nitroquinoline oxide (Ikenaga *et al.*, 1975). However, only mutations in *uvrA* or *uvrB* completely abolish incision of damaged DNA *in vivo* (Shimada *et al.*, 1968; Rupp and Howard-Flanders 1968). Because of this the *uvrC* gene was assigned a pre-excision role, where it was thought to prevent the resealing of 3'-OH, 5'-P incisions by DNA ligase (Kato 1972; Seeberg 1975; Seeberg and Strike 1976). *UvrC*$^-$ strains slowly accumulate single strand breaks but the number of breaks never reaches that in a wild type strain. However, the addition of a ligase inhibitor or an additional ligase mutation, did not affect the number of breaks in a *uvrC* dependent manner, suggesting that it was not involved in the prevention of re-ligation (Seeberg *et al.*, 1980).

The predominant UV endonuclease activity in *E.coli* is ATP dependent (Waldstein *et al.*, 1974) and Mg^{2+} requiring (Seeberg and Strike 1976) with a minor component being ATP and Mg^{2} independent (Grossman *et al.*, 1975; Braun and Grossman 1974). The major component is absent from *uvrA*$^-$, *uvrB*$^-$ and *uvrC*$^-$ strains (Shimada *et al.*, 1968; Ben-Ishai and Sharon 1978; Waldstein *et al.*, 1974) whereas the minor component is absent from *uvrA*$^-$ and *uvrB*$^-$ strains but is present in *uvrC*$^-$ strains (Braun and Grossman 1974; Seeberg and Strike 1976). The major component is recreated by mixing cell free extracts from any two of the three *uvr*$^-$ strains (Seeberg and Strike 1976) and was used as the basis for purification of the *uvrA* gene product and a *uvrB/uvrC* gene product complex (Seeberg 1978a) both of which had molecular weights greatly exceeding that reported for the minor component (Braun *et al.*, 1976) which is therefore not the product of the *uvrA* or *uvrB* genes (Seeberg 1978a). The ATP dependence of the incisions produced by the three semi-pure gene products when mixed appears to be due to the *uvrA* protein which requires ATP or GTP for binding to superhelical DNA. The binding occurs preferentially on UV irradiated DNA and is required for efficient incision (Seeberg 1978b). Further characterization of the genes and gene products proceeded after several groups had isolated the genes by cloning them into a variety of plasmids and bacteriophage. In each case reintroduction of the plasmid or phage into the corresponding *uvr*$^-$ strain restored the UV resistance of the strain and its incising capability (Rupp *et al.*, 1978; Sancar and Rupp 1979; Sancar *et al.*, 1981a, 1981b, 1981c, 1981d; Van Sluis and Pannekoek 1978; Yoakum *et al.*, 1981; Ohta *et al.*, 1981).

The gene products were identified in maxicells, which synthesise only plasmid proteins (Sancar *et al.*, 1979) or by successive inactivation of the plasmid genes with transposons and correlating loss of protein species with loss of ability to complement the corresponding *uvr*$^-$ strain (Sancar and Rupp 1979; Sancar *et al.*, 1981a). The three gene products were found to be single polypeptides of molecular weights 114000 (UVRA), 84000 (UVRB) and 70000 (UVRC) (Sancar *et al.*, 1981a, 1981b, 1981c). Overproduction of these proteins was achieved by subcloning the genes into plasmids containing powerful promoters, or by placing the relevant plasmids in a background which over-produces the proteins directly as a result of increased plasmid numbers (Rupp *et al.*, 1982). This allowed extensive purification of the gene products to examine their individual activities. No single protein or combination of two proteins has UV endonuclease activity whereas all three together form an ATP dependent, Mg^{2+} dependent UV endonuclease. This confirms that the *uvr* genes are primarily responsible for the major UV endonuclease activity *in vivo* and not for the minor component described previously (Sancar and Rupp 1983; Braun and Grossman 1974). The reason for the apparent dependence of the minor UV endonuclease activity on functional *uvrA* and *uvrB* genes is not known at present. Although all three UVR proteins are required for UV incision, the *uvrC* protein alone appears able to incise DNA in response to some DNA damaging agents. Tang and Lieberman (1982) found that it will incise N-hydroxyaminofluorine treated DNA which produces N-(guanin-(8-yl)-2-aminofluorine) as the predominant DNA adduct. The *uvrC* protein may also have AP

endonuclease activity (Sharma *et al.*, 1981; Shultz *et al.*, 1983). It also appears that the *uvrC* protein purified earlier as a *uvrB/uvrC* protein complex is not the true *uvrC* protein. The true *uvrC* protein passing through the ion exchange column which bound the *uvrB/uvrC* complex. The *uvrC* activity in the complex has been explained as a modified form of the *uvrB* protein which obviates a true *uvrC* protein requirement. The modification is *uvrC* dependent however and is absent in a *uvrC*$^-$ strain (Seeberg *et al.*, 1982).

Examination of the site of the incision of the UVRABC complete enzyme on UV irradiated DNA of known sequence unexpectedly revealed that two incisions were produced, one on either side of the damage and not one, 5' to the damage as was previously thought (Grossman *et al.*,1975; Hanawalt *et al.*,1979; Sancar and Rupp 1983). The enzyme will also incise DNA damaged with cis-platinum, psoralen plus near UV, or in response to thy(6,4) pyo adducts. The two incisions are either 12 or 13 nucleotides apart, depending on the type of damage, and both have 5'-P, 3'-OH termini. The enzyme cleaves the eighth phosphodiester bond 5' to a thymine-thymine dimer or other damage but either the fourth or fifth phosphodiester bond 3' to a thymine-thymine dimer, or a cis-platinum adduct. The 3' incision is at the fourth bond for CC, CT or TC dimers and the fifth for psoralen adducts. The reason for the variability is unknown. It has been suggested that the reason for the two widely spaced incisions is to displace the active centres of the enzyme away from DNA distortions in the immediate vicinity of the DNA damage. These distortions vary with different lesions which may alter the accessibility of adjacent phosphodiester

bonds to the enzyme active site. This is consistent with the broad substrate specificity of the enzyme and the notion that it recognises gross distortions in DNA rather than particular forms of DNA damage (Hanawalt and Haynes 1965; Sancar and Rupp 1983). However, recognition of DNA distortions must be strictly controlled since DNA is distorted for example in replicating regions and during transcription.

Another surprising property of the enzyme, in the light of previously accepted models of excision repair (Hanawalt *et al.*, 1979, 1982) is that the 12-13 nucleotide fragment is removed by the pure enzyme in the absence of exonucleases or any other cofactor. The mechanism of the removal would appear to be a combination of the natural instability of the fragment, which is not base paired in the vicinity of the dimer (Kelly *et al.*, 1969a; Hayes *et al.*, 1971) and the competitive binding of the *uvrA* and *uvrC* proteins to the resulting single-stranded region (Seeberg and Steinium 1982; Sancar *et al.*, 1981c). The *uvrB* protein also binds to a *uvrA*-DNA complex (Kacinski and Rupp 1982). The dual excision and incision capabilities of the UVRABC enzyme has led to the proposition that it be renamed as an "exinuclease" to distinguish its action from other endonucleases all of which are thought (at the moment) to make one incision (Sancar and Rupp 1983). The existence of 'exinuclease' activity has had profound consequences for the accepted models for removal of DNA damage in *E.coli in vivo* (see later).

Other examples of broad-spectrum damage-specific DNA endonucleases probably exist in yeast and mammalian cells because inactivation of any one of several different genes

(6 in yeast and possibly 7 in humans) causes a failure to incise DNA containing damage which is characteristically recognised by the UVRABC enzyme of *E.coli* (Wilcox and Prakash 1981; Reynolds and Friedberg 1981; Bootsma 1978; Hanawalt *et al.*, 1979), and because the method of incision in response to pyrimidine dimers in human cells is apparently not *via* glycosylase action (LaBelle and Linn 1982). However, the relationships between the genes is unclear as is the number actually producing structural components of a UV endonuclease. Also attempts to identify a UV endonuclease towards pyrimidine dimers in cell free extracts have consistently failed. A UV endonuclease activity has been isolated from calf thymus which is similar to the UVRABC endonuclease in having a large molecular weight and an Mg^{2+} requirement but it has not been characterised because of its exceptional lability (Waldstein *et al.*, 1978, 1979). Two low molecular weight enzymes have been isolated from *E.coli* which together probably constitute the ATP independent UV endonuclease activity seen in all *uvr*$^-$ strains (Braun and Grossman 1974; Waldstein *et al.*, 1974; Seeberg and Strike 1976) since they incise UV irradiated DNA independently of ATP and divalent cations. The smaller enzyme appears to incise in response to pyrimidine dimers (Braun and Grossman 1974; Braun *et al.*, 1975, 1976). A similar activity appears to be present in excision repair-deficient yeast strains (Bekker *et al.*, 1980), but it is unclear whether it is recognising pyrimidine dimers or some other UV photoproduct. This also applies to the other small *E.coli* UV endonuclease and a variety of other poorly characterized activities isolated from a number of sources (Waldstein 1978).

A series of UV endonuclease activities have been isolated from *E.coli* (Gates and Linn 1977a), *M.luteus* (Schön-Bop *et al.*, 1977; Hagen *et al.*, 1978; Riazuddin *et al.*,1977a,b), rat liver (Van Lanker and Tomura 1974), calf thymus (Bacchetti and Beune 1975) and human lymphoblasts (Brent 1975, 1976) which are known to recognise UV-photoproducts other than pyrimidine dimers. They all are active against DNA damaged with UV, X-rays, low pH or osmium tetroxide and probably incise in response to pyrimidine hydrates such as 5,6 dihydroxydihydrothymine (see Appendix), which is produced by all these agents (Cerutti 1974; Hariharan and Cerutti 1977; Waldstein *et al.*, 1978). Similarly, an endonuclease activity specific for 8-(2 hydroxyl 2 propyl) purines, which is also produced by UV and gamma radiation, has been isolated from *M.luteus* (Livneh *et al.*, 1979a). However, it is not known whether incisions produced by these activities are due to endonuclease action or to glycosylase action and base excision repair. Endonuclease III of *E.coli* appears representative of the non-pyrimidine dimer UV endonucleases in several respects and operates via base excision repair which suggests that the majority of the remaining activities may also (see "base excision repair"). Endonuclease V of *E.coli* appears to be unlike the majority of these enzymes in having a sharp pH optimum of 9.2-9.5, in recognising uracil-containing DNA and apurinic sites and in its ability to slowly degrade undamaged DNA (Gates and Linn 1977a; Demple *et al.*, 1980, 1982). It also appears to be a true endonuclease since it releases oligonucleotides and not free bases (Gates and Linn 1977a).

(b) Base excision repair

"Base excision repair" is distinguished from "nucleotide

excision repair" primarily by the fact that the endonuclease incision at the beginning of an excision repair event is preceded by the action of a DNA glycosylase which removes the modified part of a nucleotide by cleavage of the sugar-base glycosyl bond in the damaged nucleotide. The specificity of the overall incision in response to particular forms of nucleotide modification is determined by the glycosylase and not by the endonuclease.

The DNA glycosylases are all small monomeric proteins that are relatively specific in comparison to the UVRABC enzyme. Although they appear to be ubiquitous (Duncan 1981) most work has centred around the eight known in *E.coli* (Lindahl 1982), which are listed in Table 1. The effect on a cell of a loss of these enzymes is in most instances not known due to the lack of appropriate mutations. However, *ung* mutations inactivate uracil DNA glycosylase which has little effect on cell viability but increases transition mutations at cytosines due to a persistence of uracil in the DNA (produced by spontaneous deamination of cytosine) (Duncan *et al.*, 1976; Duncan and Miller 1980; Warner *et al.*, 1981). However, *tag* mutants, deficient in 3 methyladenine glycosylase 1, are more sensitive to the lethal effects of agents (such as MMS) that produce 3 methyladenine in DNA (Karran and Lindahl 1980).

In addition to the glycosylases described in Table 1, *E.coli* may also possess a pyrimidine dimer DNA glycosylase within the UVRABC enzyme since 10-20% of thymine-thymine dimers appear to be removed as free bases (after photoreversal) in wild type *E.coli* but not in *uvrA*$^-$ or *uvrB*$^-$ strains (Linn

Table 1 DNA glycosylases of *Escherichia coli*

Glycosylase	Substrate	Gene	Source of Damage
Uracil DNA glycosylase	uracil 5 fluorouracil	*ung*	Deamination of cytosine
Hypoxanthine DNA glycosylase	hypoxanthene	-	Deamination of adenine
3 Methyladenine DNA glycosylase 1	3 methyladenine 3 ethyladenine	*tag*	Methylating agents 5 adenosylmethionine alk.
3 Methyladenine DNA glycosylase 2	3 methyl adenine 3 methyl guanine 7 methyl adenine 7 methyl guanine	-	Methylating agents 5 adenoxylmethionine alk.
Formamidopyrimidine DNA	2,6, diamino-4-oxy-5-N-methyl formamido-pyrimidine	-	Imidazole ring cleavage of 7-methylguanine
Urea DNA glycosylase	N-substituted urea derivatives eg. formyl urea	-	X-ray fragmentation of nucleotides
Thymine glycol DNA glycosylase (endonuclease III)	5,6 dihydroxydihydrothymine 5,6 dihydrothymine	-	UV; X-rays OsO_4
Pyrimidine dimer DNA glycosylase (T_4)	pyrimidine dimers (TT TC CT CC)	denV	UV

Source:- Duncan (1981); Lindahl (1979, 1982).

et al., 1981; Bonura *et al.*, 1982). This activity has some features in common with the small molecular weight *E.coli* pyrimidine dimer UV endonucleases described earlier, although no link has been demonstrated.

The three well-characterized glycosylases that are known to recognise UV-photoproducts are described in more detail below.

The *Micrococcus luteus* pyrimidine dimer glycosylase

A UV-specific DNA degrading activity was first reported in crude extracts of *M.luteus* by Strauss (1962). Carrier and Setlow (1966) later demonstrated that the activity could remove pyrimidine dimers from DNA in the absence of any co-factors. However, purification of the activity resolved a UV endonuclease activity and a Mg^{2+} dependent "UV exonuclease" which cooperatively excised pyrimidine dimers leaving a 4-5 nucleotide single strand gap in the DNA (Nakayama *et al.*, 1967; Grossman *et al.*, 1968; Takagi *et al.*, 1968; Kaplan *et al.*, 1969, 1971). The UV endonuclease was further separated into two species having different isoelectric points and chromatographic properties (Nakayama *et al.*, 1971) and then further separated into five species by chromatography (Riazuddin and Grossman 1977a). Three of the five were active against non-pyrimidine dimer photo-products (Grossman and Riazuddin 1978). The remaining two appeared to produce 3'-OH, 5'-P termini 5' to any pyrimidine dimer but different degrees of single strandedness at these termini. They also appeared to act at different conforma-tional sites (Riazuddin *et al.*, 1977a). Despite this a mixture of both these "correndonucleases" was used on UV irradiated

DNA of defined sequence to confirm the sites of the incisions relative to pyrimidine dimers. The resulting fragments were approximately one nucleotide longer on 3' ends than expected due to the presence of a baseless nucleotide (an AP site). The AP site was found to be generated by a glycosylase which specifically cleaved the glycosyl bond of the 5' pyrimidine in a dimer and left the 3' pyrimidine untouched (see Fig.5). The glycosylase could be purified as a single protein, the two "correndonucleases" being explained as a manifestation of the unwitting purification of two AP endonucleases, both of which can act after the glycosylase (Haseltine *et al.*, 1980). The incision produced by the AP endonuclease which acts in conjunction with the UV-glycosylase produces a 3' deoxyribose with a 3'-OH terminus which is not a substrate for the 3'→5' exonuclease of DNA polymerase 1 or the polymerase activity. The appropriate substrate is produced if an additional incision is made 5' to the AP site by another AP endonuclease thereby allowing completion of excision repair (Gordon and Haseltine 1981).

The glycosylase activity cannot be purified free from all AP endonuclease activity which is likely to be because the two activities are part of the same polypeptide (Haseltine *et al.*, 1980; Grafstom *et al.*, 1981). The glycosylase is much smaller (MW15000) than the UVRABC enzyme and is a single polypeptide with a wide pH range (6.5-8.0) for activity. Although not requiring divalent cations (unlike the UVRABC enzyme) its activity is stimulated sevenfold by Mg^{2+} ions (Grafstrom *et al.*, 1981).

The repair-deficient strains of *M.luteus* isolated in the past give a confusing picture of the biological relevance of

the UV glycosylase. Takagi *et al.* (1968) concluded that the UV-incising activity is not required *in vivo* for UV resistance since all of nine mutants isolated as UV sensitive and host-cell reactivation (Hcr) minus had UV endonuclease activity. Similarly, a strain, 1312, isolated as lacking UV-endonuclease activity was UV resistant and Hcr^+ (Okubo *et al.*, 1967). Several mutants have been isolated by Grossman and co-workers, but all were selected for mitomycin C sensitivity in the mistaken belief that the UV-incising activity was similar to that of *E.coli* in having a broad range of substrates (Grossman *et al.*, 1967, 1968, 1978; Mahler *et al.*, 1971). In fact the *M.luteus* UV glycosylase appears to be specific for pyrimidine dimers (Gordon and Haseltine 1980), suggests that the mitomycin C sensitivity reflected the loss of other repair enzymes, perhaps a broader spectrum endonuclease similar to the UVRABC enzyme. Similarly the variety of phenotypes of strains obtained by transformation of the UV-sensitive mitomycin C-sensitive strain of Mahler *et al.* (1971) and Grossman *et al.* (1978) would also be consistent with the existence of more than one repair endonuclease since UV resistance and mitomycin C resistance could be restored without restoration of the "UV endonuclease" activity attributable to the UV glycosylase. The UV-sensitive, mitomycin C-sensitive DB-7 strain of Grossman *et al.* (1978) can also be restored to wild type resistance to UV in two stages but the actual deficiencies in the strain (DB-200) of intermediate resistance is confusing since it lacks a "correndonuclease" activity which is now attributable to one of the resident AP endonucleases (Haseltine *et al.*, 1980), but retains the other "correndonuclease"

(AP endonuclease) activity. The missing "correndonuclease" is restored along with UV resistance and mitomycin C resistance in strain DB-400 obtained by a single transformation of DB-200.

The T4 pyrimidine dimer glycosylase

The realisation that T4 possesses a method for repairing UV-damaged DNA stems from the observation of Luria and Dulbecco (1947) and Luria (1949) that T4 is twice as resistant to UV irradiation as phage T2. The additional resistance was found to be absent from strains mutant in gene V (Harm 1963) due to their inability to excise pyrimidine dimers (Setlow and Carrier 1966). T_2 cannot excise pyrimidine dimers (Yasuda and Sekiguchi 1970). A UV specific enzyme coded for by T4 was identified by Takagi *et al.* (1968) which would excise pyrimidine dimers in cell free extracts (Sekiguchi *et al.*, 1970). The enzyme was identified as a UV-endonuclease (Friedberg and King 1969, 1971; Yasuda and Sekiguchi 1970). Mutations in gene V renamed *denV* (Wood and Revel 1976) eliminated the endonuclease activity (Sekiguchi *et al.*, 1970; Sato and Sekiguchi 1976). The enzyme has been purified (Yasuda and Sekiguchi 1970; Friedberg and King 1971; Yasuda and Sekiguchi 1976; Friedberg *et al.*, 1980; Seawell *et al.*, 1981) and is a single polypeptide of 16800 molecular weight (Bonura *et al.*, 1982) with a broad pH optimum between pH 7-9 that acts independently of all cofactors. It is specific for pyrimidine dimers and like the *M. luteus* enzyme was originally thought to produce 3'-OH, 5'-P termini 5' to a dimer (Yasuda and Sekiguchi 1970; Friedberg and King 1971; Minton *et al.*, 1975; Nishida *et al.*, 1976; Ito and Sekiguchi 1976). Prompted by work on *M. luteus*

the T4 enzyme was also found to be a pyrimidine dimer glycosylase with an associated AP endonuclease activity residing in the same polypeptide (Demple and Linn 1980; Gordon and Haseltine 1980; Seawell *et al.*, 1980; Nakabeppu and Sekiguchi 1981). The AP endonuclease is similar to that acting after the *M.luteus* UV glycosylase in producing a 3'-OH, and 5'-P 3' to the AP site (Mosbaugh and Linn 1982; Bonura *et al.*, 1982). A second incision 5' to the AP site is therefore required to allow polymerase action as in *M.luteus*. There is no preference for the associated AP endonuclease to act after the glycosylase since the glycosylase dissociates after it has acted. Since there is no reason why a class II (see later) AP endonuclease should not incise in response to the AP site thereby creating a polymerase substrate in one step, it may be that the associated AP endonuclease is redundant *in vivo* (Bonura *et al.*, 1982).

Although the UV glycosylases are fundamentally different from the *E.coli* UVRABC enzyme they will complement the deficiency in a *uvrA*$^-$ permeabilised *E.coli* strain (Shimuizu and Sekiguchi 1979) or in a viable *uvrA*$^-$ strain when present on a plasmid (Lloyd and Hanawalt 1979) producing enhanced UV resistance.

Endonuclease III - a UV glycosylase

This enzyme was originally identified by Radman (1976) as an endonuclease that acts on heavily UV-irradiated, but not lightly UV-irradiated DNA. It also acts after pyrimidine dimers have been removed by photoreactivation. The enzyme was purified by Gates and Linn (1977a,b) and found to produce one incision for every 85 pyrimidine dimers in UV irradiated DNA

and none if the DNA contained only pyrimidine dimers. The enzyme also attacks DNA damaged by a variety of agents that produce thymine glycols such as 5,6 dihydroxydihydrothymine, which are also produced by UV radiation. Demple *et al.* (1980) showed that the incisions were the result of a glycosylase specific for thymine glycols acting in concert with an AP endonuclease, which Gates and Linn (1977) have identified as probably being part of the same molecule and which appears analogous to those associated with the pyrimidine dimer glycosylases in its failure to produce a substrate for DNA polymerase action. The enzyme appears to be the same as those previously identified as the *E.coli* "X-ray endonuclease" (Strinste and Wallace 1975; Armel *et al.*, 1977) and the urea DNA glycosylase identified by Breimer and Lindahl (1980) (Katcher and Wallace 1983). The enzyme is also similar to the pyrimidine dimer glycosylase systems in being small molecular weight (approximately 13000) and acting independently of cofactors (Katcher and Wallace 1983). The biological role of the enzyme is unknown due to the lack of appropriate mutants.

Apurinic/Apyrimidine (AP) endonucleases

These enzymes have an important role in the excision repair of various DNA lesions *via* the DNA glycosylases described above and also operate in their own right as apurinic/apyrimidinic (AP) sites arise in DNA by spontaneous base loss. The latter arise with an increased frequency when a base is modified by certain chemical additions (Lawley and Warren 1976) but the predominant source is probably *via*

glycosylase action (Lindahl 1979). A small number are generated by UV irradiation (Menshonkova *et al.*, 1980; Ljunquist *et al.*, 1974) and X-rays (Ljunquist *et al.*, 1974).

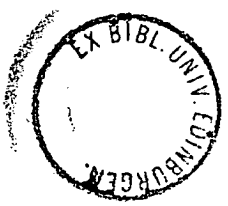
The AP endonucleases are classified according to the site and structure of the incision which they produce in response to the site of base loss. AP endonucleases that produce 3'-OH, 5'-P termini 3' to the AP site are "class 1" AP endonucleases, whereas those which produce 3'-OH, 5'-P termini 5' to the AP site are "class 2" AP endonucleases. Although other termini are theoretically possible, all known AP endonucleases fall into these two groups (Linn *et al.*, 1981). All the AP endonucleases associated with DNA glycosylases are class 1 enzymes. Twenty or so AP endonucleases have been identified to date (Friedberg *et al.*, 1981) some of which are multifunctional enzymes. Of the five known in *E.coli* more than 90% of the cell AP endonuclease activity is due to exonuclease III (also called endonuclease II or VI) which apart from its AP endonuclease activity (class 2) also possesses an RNase, a DNA 3' phosphatase and a 3'→5' exonuclease (reviewed by Weiss 1981). The majority of the remaining activity is also due to a class 2 enzyme, endonuclease IV which does not have any other catalytic activities (Gates and Linn 1977b; Ljunquist 1977). The remaining activity appears to be due to the two class 1 endonucleases III and V described earlier. The predominance of class 2 enzyme activity would suggest that the AP sites generated by endonuclease III may be attacked by these enzymes in preference to the associated AP endonucleases *in vivo* as was suggested for the T4 UV glycosylase-generated AP sites (Bonura *et al.*, 1982).

The biological roles of the enzymes is difficult to assess due to the redundancy of the AP endonucleases in *E.coli* and the lack of strains defective in various combinations of the AP endonucleases. Thus *xth*$^-$ strains (lacking exonuclease III) are only slightly more sensitive to agents that generate AP sites *via* the damage-specific glycosylases, for example MMS which generates AP sites *via* 3 methyladenine glycosylases (Ljungquist *et al.*, 1976). However, when extremely large numbers of AP sites are generated in a *dut*$^-$ strain causing overincorporation of uracil into DNA which is then removed by uracil DNA glycosylase, a lack of exonuclease III leads to reduced survival (Taylor and Weiss 1982; Warner *et al.*, 1981).

The removal of lesions from DNA by excision repair

The early work on excision repair of pyrimidine dimers in *E.coli* gave rise to several models for how the lesion was removed after the incision event (reviewed in Setlow 1968). The model which became generally accepted visualised specific exonucleases which would processively degrade away from an incision in the direction of the damage thereby releasing it from the DNA. An alternative model which proposed that the damage is removed in a single step without the use of exonucleases, leaving a small single strand gap, was largely ignored in favour of the former for several reasons. Excision of pyrimidine dimers was found to be accompanied by DNA degradation which was greater than could be accounted for by the loss of damaged nucleotides alone (Boyce and Howard-Flanders 1964). Also enzyme extracts from *M.luteus* which

UV irradiated DNA would only excise pyrimidine dimers when an additional enzyme, the "UV exonuclease" was present (Grossman *et al.*, 1968). The discovery of 'nick translation' from an internal DNA break (ie an incision) by DNA polymerase 1 and its associated 5'→3' exonuclease provided a convenient mechanism by which this could occur in *E.coli* (Kelly *et al.*, 1969a, 1969b; Cozzarelli *et al.*, 1969; Wu 1970; Dumas *et al.*, 1971) despite the fact that *polAex* mutants (lacking the 5'→3' exonuclease) were excision proficient (Cooper and Hunt 1978), although they were deficient in the restoration of *uvr* induced strand breaks in a similar manner to *polA*$^-$ strains (Sharon *et al.*, 1975). Measurement of the size of the excised region at 20-30 nucleotides (Cooper and Hanawalt 1972a; Rothman 1978) tended to support the idea that a region of DNA around the damage was degraded by exonucleases. The imagined 'nick translation' of the damaged region was also attractive because it required only one incision 5' to the damage and all three UV DNA damage-incising activities known appeared to incise 5' to the damage generating 3'-OH 5'-P groups which is a substrate for DNA polymerase 1 (Braun and Grossman 1974b; Braun *et al.*, 1976; Grossman *et al.*, 1978; Riazzudin and Grossman 1977b). Also, the 5'→3' exonuclease of DNA polymerase 1 was found to degrade UV irradiated DNA in such a manner that small oligonucleotides (observed as the products of pyrimidine dimer excision *in vivo*) were produced (Kelly *et al.*, 1969b). The failure to implicate the 5'→3' exonuclease of DNA polymerase 1 genetically in the excision process was taken to be due to its redundancy, since several other exonucleases could theoretically substitute for the 5'→3' exonuclease of DNA polymerase 1,



notably the 5'→3' exonuclease of DNA polymerase III, exonuclease VII and exonuclease V all of which degrade UV irradiated DNA (Kelly *et al.*, 1969b; Chase and Richardson 1974; Livingston and Richardson 1975; Tanaka *et al.*, 1975). Strains lacking exonuclease VII (Chase and Richardson 1977) or exonuclease V (Schlaes *et al.*, 1972) had excision characteristics similar to the wild type which was also taken as evidence of their redundancy and was supported by the slower excision in strains lacking the 5'→3' exonuclease of DNA polymerase 1, exonuclease VII and exonuclease V (Masker and Chase 1978). The involvement of exonuclease V was implied because excision attributable to exonuclease VII only occurred in the absence of exonuclease V and also because strains lacking exonuclease V degraded less DNA after UV irradiation than the wild type (Masker and Chase 1978).

The finding that the UVRABC enzyme can remove the damaged region alone *in vitro* (Sancar and Rupp 1983 see earlier) renders it unlikely that these exonucleases play a major role in excision *in vivo* although they may be involved to some extent. The alternative model for excision was therefore correct against the weight of the available evidence.

The *uvrD* gene appears to have a more profound effect on excision repair than any exonuclease. *uvrD*$^-$ strains are UV sensitive and MMS sensitive and in some cases degrade their DNA excessively after UV irradiation (Ogawa *et al.*, 1968). A *uvrB*$^-$ *uvrD*$^-$ strain is more UV sensitive than a *uvrD*$^-$ strain suggesting that *uvrD* functions after incisions have taken place (Ogawa *et al.*, 1968) *uvrD* mutations do not eliminate UV-induced incision (Shimada *et al.*, 1968) but reduce the number

of incisions made by the UVRABC enzyme (Ben-Ishai and Sharon 1981) and produce a marked inability to excise pyrimidine dimers (Kuemmerle and Masker 1980) and to perform host-cell reactivation (Ogawa *et al.*, 1968). *uvrD* also has a role in genetic recombination (Horii and Clark 1973; Arthur and Lloyd 1980; Howard-Flanders and Bardwell 1981), transposon excision (Llunblad and Kleckner 1982), spontaneous mutation (Smirnov *et al.*, 1972), induction of multiple linked mutations (Sklar 1980) and DNA replication (Horiuchi and Nagata 1973; Siegel 1973; Smirnov *et al.*, 1973). The *uvrD* gene has also been called *mutU* (Siegel 1973), *uvrE* (Smirnov and Skavronskaya 1971) and *recL* (Horii and Clark 1973) which are all *uvrD* alleles (Kushner *et al.*, 1978; Maples and Kushner, 1982).

The *uvrD* gene product is DNA helicase II (Arthur *et al.*, 1982; Maples and Kushner 1982; Oeda *et al.*, 1982; Hickson *et al.*, 1983) an ATP-dependent DNA helicase which promotes the melting of DNA (or RNA which is annealed to single stranded DNA) (Abdel-Monem *et al.*, 1977a, 1977b). It would seem reasonable to assume therefore that DNA helicase II is at least partially responsible for the removal of the 12-13 nucleotide fragment generaged by the UVRABC enzyme *in vivo* but not necessarily *in vitro*. Further support for this idea is that *uvrD* mutations decrease the short patch component of excision repair (see later) which is thought to be the component produced by the UVRABC enzyme (Youngs and Smith 1976; Sancar and Rupp 1983). It has been suggested that helicase and the UVRABC enzyme could form a complex which irreversibly binds to DNA when *uvrD* is mutant (Ben-Ishai and Sharon 1981).

The requirement for DNA helicases in the repair of DNA in other systems may be similar where enzymes similar to the UVRABC enzyme are found but their requirement in the repair of UV glycosylase mediated incisions is unknown. Repair in this case may still proceed *via* the classical model of excision repair and require exonuclease action. Indeed T4 (Shimuzu and Sekiguchi 1976) and *M.luteus* (Hamilton *et al.*, 1974) both produce at least two suitable exonucleases which degrade UV-irradiated DNA. The exonucleases would release the pyrimidine dimer which after UV glycosylase action is still attached to the DNA by the 3' nucleotide of the dimer. This is supported by the fact that 4-7 nucleotides are removed for each pyrimidine dimer in T4 (Bonura *et al.*, 1982).

Repair of AP sites in DNA in most other instances can proceed *via* three routes. The missing base can be replaced by an insertase (Livneh *et al.*, 1979b; Livneh and Sperling 1981), or the baseless phosphate removed by 3'→5' or 5'→3' exonuclease action, depending upon whether the incision is by a class 1 or class 2 AP endonuclease respectively (Linn *et al.*, 1981; Verly 1982) or by the concerted action of a class 1 and class 2 enzyme acting in sequence (Linn *et al.*, 1981; Verly 1982). All three processes have been demonstrated *in vitro* but the situation *in vivo* is not known.

Refilling of gaps - Polymerases and Patch size

After lesions have been removed by degradation of a region of DNA encompassing the lesion, the resulting single-strand gap must be filled by polymerase action. In *E.coli* DNA

polymerase 1 is primarily responsible for the resynthesis although DNA polymerases II and III are capable of repair resynthesis but appear to be not involved unless DNA polymerase I is absent (Masker *et al.*, 1975; Cooper 1982). DNA polymerase I is probably more suited to repair resynthesis because it can bind at an incision whereas the remaining polymerases require single stranded regions in excess of 20 nucleotides (Kornberg 1981). Measurement of the size of the resynthesised region (a patch) in *E.coli* after UV irradiation reveals that the majority (99%) are short and approximately the size of the fragment removed by the UVRABC enzyme, the remainder being more than 1500 nucleotides in size and may be the result of a failure of short patch repair to occur in specialised regions of the chromosome such as in transcribing or replicating regions (Cooper and Hanawalt 1972a, 1972b; Cooper 1982). The synthesis of the smaller patches can occur in the absence of protein synthesis and does not require energy whereas the long patch synthesis requires protein synthesis and ATP (Smith *et al.*,1978). Long patch production is also unlike short patch production in its requirement for functional *recA*, *lexA*, *recB*, *recF* and *uvrD* genes (Smith *et al.*, 1978; Hanawalt *et al.*, 1982), which indicates that it is part of the SOS response (Cooper 1982). However the roles of these gene products have not yet been elucidated. Both long and short patches produced in the repair of UV damage depend on the UVRABC enzyme (Cooper and Hanawalt 1971). Repair of AP sites and a variety of other damage appears to be *via* short patch resynthesis (Regan and Setlow 1974; Karran *et al.*, 1977; Hanawalt *et al.*, 1982) and may be as short as one nucleotide if an AP site is excised by a combination of a

class 1 and class 2 AP endonuclease (Livneh and Sperling 1981; Lindahl 1982). The patches produced in T4 and *M.luteus* in response to UV glycosylase action appear to be *via* a short patch resynthesis of under 10 nucleotides and presumably carried out by one of the equivalent DNA polymerase I type enzymes produced by these organisms (Grossman *et al.*, 1968, 1975; Bonura *et al.*, 1982).

DNA ligase

DNA polynucleotide ligase catalyses the covalent joining of juxtaposed 3'-OH and 5'-P groups at single-strand breaks in duplex DNA (Olivera and Lehman 1967) and is considered essential for the resealing of the break left after the resynthesis step of excision repair (see fig.5) (Youngs and Smith 1977). DNA ligase mutants are UV sensitive (Pauling and Hamm 1968) and DNA ligase has been shown to participate in the repair of UV damage *in vitro* (Heijneker *et al.*, 1971) and to control the extent of repair replication after UV irradiation (Pauling and Hamm 1968; Youngs and Smith 1977).

CHAPTER 2

Materials and Methods

Bacteria and bacteriophages

The strains of *Deinococcus radiodurans* used are listed in table 2 with their relevant phenotypes. Other bacteria and the bacteriophage used are listed in table 3. All of the strains in table 2 failed to grow on TGY agar containing rifampicin (1µg ml^{-1} Sigma). Rifampicin-resistant clones of these strains were obtained by plating approximately 10^8 bacteria onto TGY agar plates containing rifampicin (50µg ml^{-1}). Typically 5 to 10 spontaneous rifampicin-resistant clones grew which would grow on TGY agar containing up to 100 µg ml^{-1} of rifampicin. The acquisition of rifampicin resistance did not affect the sensitivity of the strains to the lethal effects of UV or mitomycin C.

Bacteria were stored on agar plates at 4°C and subcultured at 4 to 6 week intervals. Bacteriophage T4 was stored at 10^{11} pfu ml^{-1} in SMG buffer at -20°C. Bacteriophage PM2 was stored in filter sterilized Q medium at 4°C.

All bacteria were grown in batch cultures on an orbital shaker at 30°C unless otherwise stated. The growth of bacteria was monitored using a nephelometer (Evans Electroselenium Ltd. Halstead) with an orange filter.

Media

The media for the growth of bacteria are listed below.

TGY medium for the growth of *D.radiodurans*, *D.proteolyticus* and *D.radiopugnans*

Tryptone (Difco) 5 $g\ell^{-1}$

Yeast extract (Difco) 3 $g\ell^{-1}$

D.glucose 1 $g\ell^{-1}$

Table 2 Strains of *D. radiodurans*

Strain	Source	Phenotype			Mutant Genes	Reference
		UV	MTC	MMS		
Wild-type R_1	-	R	R	R		a
301	MTC sensitive derivative of 303 selected after MNNG mutagenesis	S	I	S	*uvsA mtcA*	b
302	UV resistant transformant of 301 obtained using R_1 DNA	R	S	R	*mtcA*	b,c
303	UV sensitive mutant of R_1 selected after MNNG mutagenesis	S	I	S	*uvsA*	b
261	derivation as for strain 301	S	I	-	*uvsB mtcA*	b
rec 30	UV sensitive mutant of R_1 selected after MNNG mutagenesis	S	S	-	*rec 30*	d
UVS9	UV sensitive mutant of 302 selected after MNNG mutagenesis	S	S	R	*uvsC mtcA*	e
UVS25	UV sensitive mutant of 302 selected after MNNG mutagenesis	S	S	R	*uvsD mtcA*	e
UVS78	UV sensitive mutant of 302 selected after MNNG mutagenesis	S	S	R	*uvsE mtcA*	e
91	MTC resistant transformant of UVS9 obtained using R_1 DNA	R	R	R	*uvsC*	f
251	MTC resistant transformant of UVS25 obtained using R_1 DNA	R	R	R	*uvsD*	f
781	MTC resistant transformant of UVS78 obtained using R_1 DNA	R	R	R	*uvsE*	f
112	UV sensitive mutant of 302 selected after MNNG mutagenesis	S	S	S	*rec-1 mtcA*	f
128	UV sensitive mutant of 302 selected after MNNG mutagenesis	S	S	S	*uvsF mtcA*	f
131	UV sensitive mutant of 302 selected after MNNG mutagenesis	S	S	S	*uvsG mtcA*	f
8	UV sensitive mutant of 302 selected after MNNG mutagenesis	S	S	S	*uvsF mtcA*	f
1281	R_1 DNA transformant of 128, intermediate MTC resistance selected	S	I	S	*uvsF*	f
1311	R_1 DNA transformant of 131, intermediate MTC resistance selected	S	I	S	*uvsG*	f

Abbreviations

R = resistant; S = sensitive; I = intermediate sensitivity; UV = ultraviolet light; MTC = mitomycin C; MMS = methyl methanesulphonate

References

a - Anderson *et al.*, 1956; b - Moseley & Copland, 1978; c - Tempest & Moseley, 1980; d- Moseley & Copland, 1975; e - Moseley & Evans, 1983; f - derived in this thesis.

The origins and further details of the relationships between the strains derived in this thesis are described in figure 16 (page 124).

Table 3 Other Bacterial Strains and Bacteriophase

Strain	Genotype	Source or reference
E.coli D5683	F^- str R Bi^- *lig*$^+$*lop*8 thy (low) (λCI857,5)	B.E.B.Moseley, Edin.
E.coli CSH25	B^- SupF ($SuIII^+$)	B.E.B.Moseley, Edin.
E.coli HB101	R^-_B M^-_B strR *thy*$^-$ *proA*$^-$ *leu*$^-$ nsdS20 *recA13 ara14 lacY1 galK2 rpsL20 xyl5 mtl-1 supE44* λ^-	(Boyer and Roulland-Dussoix 1969) M.Mackay, Edin.
E.coli B/C2	-	B.E.B.Moseley, Edin.
E.coli B/C2/HT	thy$^-$ (high)	This thesis
E.coli B/C2/LT	thy$^-$ (low)	This thesis
Deinococcus radiophilus	-	B.E.B.Moseley, Edin.
Deinococcus proteolyticus	-	B.E.B.Moseley, Edin.
Deinococcus radiopugnans	-	B.E.B.Moseley, Edin.
Alteromonas espejiana BAL 31	-	(Hurley and Deering 1981)
bacteriophage PM2	-	(Hurley and Deering 1981)
bacteriophage T4	-	I.W.Dawes, Edin.

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Table 4 Plasmids

Plasmid	Antibiotic resistance marker	Reference
pML2	kanamycin	Hershfield *et al.*, 1974
pAT153	ampicillin tetracycline	Twigg and Sherrat 1980

Both plasmids were resident in *E.coli* HB101

M9 medium for the growth of *E.coli* B

sterile M9 salts x 10	10 ml
sterile 20% glucose	1 ml
sterile 0.1M$MgSO_4$	1 ml
sterile distilled water	88 ml

M9 salts x 10

Na_2HPO_4	6 $g\ell^{-1}$
KH_2PO_4	3 $g\ell^{-1}$
NaCl	0.5 $g\ell^{-1}$
NH_4Cl	1 $g\ell^{-1}$

M9-CA medium for the growth of *E.coli* D5683 is M9 medium with the addition, just prior to use, of 0.1 ml of filter sterilized aneurine HCl (2 $mgml^{-1}$), 1 ml 15% casamino acids (Difco) and 6µl of thymine (1 $mgml^{-1}$).

LBP medium for the growth of *E.coli* HB101

Tryptone (Difco)	5 $g\ell^{-1}$
NaCl	0.5 $g\ell^{-1}$
1M NaOH	2 ml ℓ^{-1}

A

10 ml of sterile 20% glucose ℓ^{-1} of A was added just prior to use.

LB medium for the growth of *E.coli* CSH25

Tryptone (Difco)	10 $g\ell^{-1}$
Yeast extract (Difco)	5 $g\ell^{-1}$
NaCl	5 $g\ell^{-1}$
1M NaOH	3.3 ml ℓ^{-1}

A

1M $MgSO_4$	

B

A and B were autoclaved separately. 10 ml B are added to 1ℓ A just prior to use.

TYM medium for the growth of *Alteromonas espejiana* BAL 31

Tryptone (Difco)	10 g ℓ^{-1}	
Yeast extract (Difco)	5 gℓ^{-1}	
NaCl	26 gℓ^{-1}	A
KCl	0.7 gℓ^{-1}	
15% $CaCl_2 2H_2O$ w/v		B
24% $MgSO_4$ $7H_2O$ w/v		C

A, B and C were autoclaved separately. 10 ml B and 10 ml C were added to A just before use.

Nutrient broth 2 for growth of *D.radiophilus* and *E.coli*

Nutrient broth 2 (Oxoid) 23 gℓ^{-1}

All media were solidified for agar plates with 15 gℓ^{-1} of bacteriological agar no.1 (Oxoid). Sloppy agar for titering phage contained 7 gℓ^{-1} of the same agar. All media were autoclaved at 15 psi for 15 min.

Buffers used

67 mM phosphate buffer pH 7.0

KH_2PO_4	4.56 gℓ^{-1}
Na_2HPO_4	4.73 gℓ^{-1}

phosphate EDTA buffer (PEB) pH 7.0

KH_2PO_4	0.52 gℓ^{-1}
K_2HPO_4	2.82 gℓ^{-1}
EDTA	3.36 gℓ^{-1}

butanol saturated PEB (BSPEB)

PEB	10 volumes
butanol-1-ol	1 volume

SMO buffer pH 7.5

pH adjusted with HCl	Tris base	2.4 $g\ell^{-1}$
	$MgCl_2$	1.5 $g\ell^{-1}$
	NaCl	5.8 $g\ell^{-1}$

STOP buffer

Sodium dodecyl sulphate	0.09 g
EDTA sodium salt	0.04 g
Bromophenol blue (Sigma)	0.02 g
Ficol (Sigma)	2.0 g
20mM Tris HCl pH 8.0	10 mls

TE buffer pH 8.0

Tris base	1.21 $g\ell^{-1}$
EDTA sodium salt	3.72 $g\ell^{-1}$

TAE buffer pH 8.2 (x10)

pH adjusted with acetic acid	Tris base	48.4 $g\ell^{-1}$
	Sodium acetate	27.2 $g\ell^{-1}$ (trihydrate)
	EDTA Na salt	4.0 $g\ell^{-1}$

Tris HCl was used routinely as a buffer at a variety of molarities and pH values and these are described as they arise. These buffers were made by dissolving the appropriate quantity of Tris base in distilled water and adjusting the pH with HCl.

Chemical reagents

Routine chemicals used were purchased from BDH Chemicals Ltd., Poole, Dorset unless otherwise indicated. The address of the main other suppliers quoted are:- Koch-Light Laboratories,

Colnbrook, Bucks: Sigma Chemical Co., London: Difco Laboratories, Detroit, Michigan: and Oxoid Ltd., Basingstoke, Hants.

Other sources are described as they arise.

DNA damaging agents

Methyl methanesulphonate (MMS) (Eastman-Kodak and Co. Rochester, New York) and ethyl methanesulphonate (EMS) (Sigma) were stored in the dark at room temperature until use. N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG) (Sigma) and N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG) (Aldrich Chemical Co., Dorset) were dissolved in 67mM phosphate buffer at 1 $mgml^{-1}$, filter sterilized, and stored in 1 ml aliquots at -20°C until use. Excess ENNG and MNNG from aliquots were disposed of into 40% sodium thiosulphate after use. Mitomycin C (Sigma) and decarbomyl mitomycin C (DCMTC - a gift from Kyoma Hakko Kogyo Co. Ltd., Tokyo) were dissolved in 50% methanol : 50% distilled water at 500μgml^{-1}, filter sterilized and stored in the dark at 4°C. Hydroxylamine hydrochloride (HA) (Sigma) was dissolved in phosphate buffer pH 5.8 (132 $mgml^{-1}$) prior to use. 4-Nitroquinoline oxide (NQO) (Eastman Kodak) was dissolved in dimethyl sulphoxide (DMSO) (Koch-Light) at 1.5 $mgml^{-1}$ and stored at -20°C.

Radioactive label

6-^{3}H thymidine (dThd) 27 Ci $mmol^{-1}$, 1$\mu Ci\ ml^{-1}$ in water was purchased from Amersham International, Bucks.

Enzymes

Enzymes were purchased from Sigma. Egg white lysozyme (grade 1) was stored at 4°C until use. DNase 1 and RNase were dissolved in distilled water at 1 $mgml^{-1}$ at -20°C until use.

Exposure of *D.radiodurans* to DNA-damaging agents

Overnight cultures of *D.radiodurans* were diluted in TGY medium to a nephelometer reading of 10. Approximately 20 ml was grown in a 250 ml flask with a side arm. Cultures were allowed to grow to a nephelometer reading of 30 which corresponds to approximately 1 x 10^8 viable units ml^{-1}. The cultures would be growing exponentially at this point.

UV irradiation. A sample of the culture was centrifuged, washed and resuspended in an equal volume of 67 mM phosphate buffer and irradiated with 254 nm UV light from a Hanovia germicidal lamp (Hanovia Lamps Ltd., Slough, Bucks) at a dose rate of 1.05 $Jm^{-2}sec^{-1}$. For measurement of survival or mutation 5 ml of the washed bacterial suspension was irradiated in a glass Petri dish with stirring. Smaller volumes were sometimes irradiated in other studies as will be described later.

Mitomycin C (or DCMTC). Samples of culture were resuspended in an equal volume of fresh TGY medium. Mitomycin C (or DCMTC) was then added to the required concentration.

Other chemicals. Samples of cells were washed twice in 67 mM phosphate buffer pH 7.0 (or 0.1M phosphate buffer, 0.1M NaCl pH 5.8 in the case of HA), and the relevant chemical added to

the required concentration. In the case of HA and EMS which dissolve relatively slowly, the chemical was dissolved in the buffer at twice the required concentration and added to an equal volume of bacterial suspension. Exposure to all chemicals was terminated by washing the cells by repeated resuspension in TGY and by dilution in some instances. Chemicals were discarded into 20% sodium thiosulphate.

Measurement of survival

The survival of cultures after exposure to UV light was routinely monitored by exposing a streak of bacteria on a TGY plate to increasing doses of UV light. Survival after exposure to all DNA damaging agents was quantified by plating serial dilutions of samples, withdrawn during exposure to an agent, on TGY agar. Viable colonies were counted after growth at 30°C for 3 days.

Measurement of survival in the presence of chloramphenicol

Chloramphenical (15µg ml^{-1}) was added to exponentially-growing cultures immediately after UV irradiation. The bacteria were resuspended in TGY containing chloramphenicol (15µg ml^{-1}) and incubated with shaking at 30°C. Samples were withdrawn at various times, diluted and plated on TGY agar. Colonies were counted after 3 days incubation at 30°C. Alternatively a plate test was used to monitor survival as shown in fig. 30. Bacterial suspensions were streaked onto Millipore filters (5cm, 0.45µm) laid onto dry TGY plates containing 10µg ml^{-1} of chloramphenicol. The plate absorbs the medium leaving the cells on the filter surface. The bacteria were then UV irradiated, incubated for 15 h at 30°C

and the chloramphenicol removed by placing the disc onto two changes of new TGY plates for 3 h. The bacteria that survived grew to form single colonies or streaks on the disc surface as nutrients passed through the filter.

Measurement of survival after two UV doses

Exponentially growing bacteria at a nephalometer reading of 30 were resuspended in 67mM phosphate buffer and a 0.1 ml sample diluted and plated on TGY agar; the remainder was UV irradiated. Two samples were withdrawn after irradiation, one of 5 ml and one of 0.1 ml. The former was added to a side arm flask containing 15 ml of 1.3 fold concentrated TGY; the latter was appropriately diluted and plated on TGY agar to provide the surviving fraction of bacteria after the first UV dose. The flask was incubated at 30°C in an orbital shaker. At various times the optical density of the culture was measured and 1.1 ml samples removed and resuspended in the same volume of phosphate buffer. 0.1 ml of the suspension was diluted and plated; the remainder was irradiated then diluted and plated on TGY agar.

Measurement of mutation induction

Cultures treated with a DNA-damaging agent were resuspended twice in TGY and serially diluted in TGY broth for viable counts. 10^2 dilutions were incubated at 30°C on an orbital shaker until the culture had reached stationary phase, samples withdrawn, serially diluted and plated on TGY agar containing 20μg ml^{-1} rifampicin or TGY agar. Rifampicin resistant colonies grew after 3 days incubation at 30°C. The frequency of rifampicin resistant colonies was then determined.

Isolation of DNA for genetic transformations

Rifampicin-resistant clones were used as the source of DNA. 5 ml of overnight cultures grown in TGY broth were used to inoculate 1.5 litres of fresh TGY medium containing 5µg ml^{-1} rifampicin. After growth for 18 h at 30°C on an orbital shaker the cultures were in stationary phase and contained approximately 10^9 viable units ml^{-1}. After centrifugation at 5000 g for 5 min the pellets (approximately 2 g wet weight) were resuspended and held for 15 min in BSPEB (50 ml) at 0°C; this removes the lipid containing wall layers which prevents lysozyme degrading the peptidoglycan (Driedger and Grayston 1970). The cells were then resuspended in PEB (50 ml) containing lysozyme (1mg ml^{-1}) and incubated at 37°C for 45 min, after which 5 ml of PEB containing 11% sodium dodecyl sulphate (SDS) was added. The cells lysed completely within 5 min producing a deep red gel. DNA was extracted from the gel essentially according to Marmur (1961). 13 ml of 5M sodium perchlorate (to stabilise the DNA) and 73 ml of chloroform/isoamylalcohol (24:1 vol/vol) were added to the gel which dispersed to a pink liquid with vigorous shaking. Centrifugation (12000 g for 30 min) of the pink liquid yields a clear aqueous upper layer containing the nucleic acids, a solid white middle layer of denatured protein, and a lower pink chloroform layer containing protein. The aqueous layer was removed using a pipette added slowly to 1.7 volumes of absolute ethanol and the precipitated DNA wound onto a glass rod and air dried. The DNA was redissolved in 10mM Tris HCl 1mM EDTA pH 8.0 (TE buffer) and re-extracted with an equal volume of chloroform/isoamylalcohol. The phases were separated by centrifugation (12000 g for 20 min) and the upper aqueous phase

removed and added slowly to 1.7 volumes of absolute ethanol. The DNA was wound out, dried and finally dissolved in TE buffer at a concentration of approximately 1 to 2 mg ml^{-1}. Additional treatment of the DNA with RNase (50µg ml^{-1} for 60 min at 37°C) did not alter the frequency of transformation and was omitted.

Preparation of labelled DNA

^{3}H DNA was obtained from a rifampicin-resistant derivative of *D.radiodurans* strain 91 (strain 91 rif^{R}) which incorporated dThd into DNA to a two to four fold greater extent than the wild type strain. Extraction of DNA was essentially as for unlabelled DNA. 0.2 ml of an overnight culture of strain 91 rif^{R} grown in TGY broth was used to inoculate 20 ml of TGY broth containing 5µg ml^{-1} rifampicin and 25µCi ml^{-1} of ^{3}HdThd. After 18 hours growth the culture was lysed as above in 5 ml of buffer. The resulting gel was deproteinised by repeated extractions with an equal volume of chloroform/isoamylalcohol but the DNA was not wound out onto glass rods. RNA was removed by treatment with RNase (50µg ml^{-1}) for 30 min at 37°C and the remaining DNA was re-extracted with chloroform/isoamyl-alcohol then precipitated with 0.6 volumes of isopropanol. The DNA was finally resuspended in 1 ml of TE buffer at a concentration of 400µg ml^{-1}. The specific activity of the DNA was 25000 cpm μg^{-1}.

Isolation of thy^{-} strains of *E.coli* B

E.coli B/C2 was grown in M9 medium to stationary phase and 1 ml plated on M9 agar containing trimethoprim (100µg ml^{-1}) and M9 agar containing trimethoprim (100µg ml^{-1}) and thymine (50µg ml^{-1}). About 200 colonies grew on the latter plate

after 2 days but none on the former plate. Isolated clones were selected by streaking onto M9 medium containing trimethoprim (100μg ml^{-1}) and thymine (50μg ml^{-1}) and these were tested for growth on M9 medium and M9 containing thymine (50μg ml^{-1}). One clone which grew only on the latter was kept as strain B/C2/HT. Low thymine requiring derivatives of B/C2/HT were selected after treatment of exponentially growing bacteria with MNNG (20μg ml^{-1}) for 10 min. The culture was washed and allowed to grow for an hour in M9 medium containing thymine (50μg ml^{-1}) after which the trimethoprim selection was repeated except that 3μg ml^{-1} of thymine was used. Strain B/C2/LT resulted from this selection and would only grow on M9 medium if it contained thymine (3μg ml^{-1}).

Phage T4 titres

Bacteriophage T4 was titered by serial dilution in SMO. 0.1 ml of dilutions was mixed with 0.1 ml of a ten fold concentrated culture of *E.coli* B/C2 resuspended in nutrient broth 2 and incubated at 37°C for 15 min. 2.5 ml of sloppy nutrient broth 2 agar at 46°C was mixed with the bacteria-phage suspension and plated on nutrient broth no.2 agar plates preheated to 37°C. Plaques appeared after 15 h growth at 37°C.

Labelling T4 DNA

3 ml of an overnight culture of *E.coli* B/C2/LT grown in M9 medium containing thymine (3μg ml^{-1}) was used to inoculate 300 ml of the same medium containing 1μCi ml^{-1} of ^{3}HdThd. The culture was grown to a nephalometer reading of 50 (approx. 1×10^{9} viable units ml^{-1}) and phage T4 added to a multiplicity of infection of 3. The culture was allowed to grow for two hours at 37°C during which time the cells lysed. The lysate

was cleared by centrifugation at 12000 g for 10 min and the pellet discarded. The supernatant was incubated for a further hour at 37°C with the addition of 12 g NaCl, 1 mg of DNase and 1 mg of RNase. 30 grams of polyethylene glycol 8000 (Sigma) was then added and dissolved and the lysate left overnight at 4°C. The precipitate containing the phage was collected by centrifugation (12000 g for 10 min) at 4°C and resuspended in SMO (10 ml). Cell debris was removed by further centrifugation (12000 g for 10 min) and the supernatant layered onto a three 1.5 ml step CsCl gradient of densities 1.3, 1.5 and 1.7 (0.87, 1.59 and 2.53 g of CsCl in 2 ml of distilled water respectively). The gradient was spun in a 3 x 15 ml MSE swing out rotor at 25000 rpm for 1 h when the T4 appears as an opaque band in the middle step. The band was extracted with a syringe through the tube side. The phage was dialysed against 10mM Tris HCl pH 7.5, 1mM EDTA overnight (or SMO if the phage was kept as stock). DNA was liberated from phage either by adding the phage directly to alkaline sucrose gradients or by liberating the DNA from the phage with an equal volume of formamide.

Phage λ titres

Phage λ isolated from *E.coli* D5683 contains a lysis mutation which is suppressed by an amber suppressor mutation in *E.coli* CSH25 which was therefore used as the indicator strain. *E.coli* CSH25 was grown to stationary phase in TYM medium and resuspended in the same volume of 10mM $MgSO_4$. The maltose induces λ receptors to be produced on the cell surface. The phage was serially diluted in SMO and 0.1 ml of dilutions

mixed with 0.1 ml of indicator bacteria at 37°C for 15 min to preadsorb the phage. 2.5 ml of sloppy agar at 46°C was mixed with this and plated on LB agar plates at 37°C. Plates were incubated at 37°C overnight because the λ contains a mutation which prevents it becoming a lysogen at this temperature.

Labelling phage λ DNA

E.coli D5683 which contains λ as a prophage was grown at 30°C overnight in M9CA medium. At this temperature the prophage will not enter the lytic cycle. 3 ml of this culture was used to inoculate 300 ml of M9CA containing deoxyadenosine (250μg ml^{-1}) and 1μCi ml^{-1} ^{3}HdThd. The culture was grown to an OD of 50 at 30°C in an orbital shaker and the flask transferred to a shaking water bath at 42°C for 30 min to induce the prophage. The temperature of the bath was turned down to 38°C and the incubation continued for a further 2 h. The phage is lysis defective and accumulates within the bacteria during this period. The bacteria were collected by centrifugation (6000 g for 4 min) and the supernatant discarded. The cells were washed and resuspended in SMO (10 ml) and permeablized by adding 10 drops of chloroform. The phage were released overnight at 4°C. Phage were further purified on CsCl step gradients as for phage T4. Phage was found at the interface between the two top steps and was either added directly onto alkaline sucrose gradients or the DNA released with formamide.

Genetic transformation

The procedure used was essentially that developed by

Tirgari and Moseley (1980). An overnight culture was diluted in fresh TGY medium to a nephelometer reading of 10 and grown to a reading of 30 at which point the bacteria were resuspended in half the volume of fresh TGY. 0.4 vol. 0.1M $CaCl_2$ was added to the culture which was subsequently held on ice for 10 min. 270µl of the chilled culture was added to 30µl of DNA (1mg ml^{-1}), incubated at 30°C with gentle shaking for 90 minutes, diluted tenfold and then allowed to grow for several hours for phenotypic expression. The culture was then diluted and plated on TGY agar and TGY agar containing rifampicin (20µg ml^{-1}) or mitomycin C (0.05µg or 0.015µg ml^{-1}). Selection for UV-resistant transformants was by irradiation of the bacteria on TGY plates which had been allowed to dry after plating. Resistant transformants were scored after incubation at 30°C for 3 days.

Isolation of UV-sensitive mutants

An exponentially-growing culture of strain 302 was grown to an OD of 30 (1×10^8 vu ml^{-1}) and resuspended in phosphate buffer pH 7.0. MNNG dissolved in phosphate buffer was added to a final concentration of 20µg ml^{-1} and the culture incubated at 30°C for 30 min. The culture was washed three times in fresh TGY broth and then allowed to grow to stationary phase. This culture was diluted and plated on 50 TGY agar plates so that approx 200 colonies grew per plate. The plates were incubated at 30°C for 2 days then replicated, using sterile 7cm filter discs (Whatman no.1) onto fresh TGY plates. The replica plates were irradiated with 380Jm^{-2} of UV and colonies allowed to grow for 2 days at 30°C. Colonies which failed

to grow on UV irradiated plates were rescued from the master plates and streaked onto TGY plates. Single clones isolated from streaks were tested for UV sensitivity.

Measurement of pyrimidine dimer content

The method used was essentially according to Moseley and Copland (1975) which relies on the differential chromatographic mobilities of pyrimidine dimers and monomers. 0.025 ml of an overnight culture of bacteria was added to 2.5 ml of TGY medium containing 50μCi ml^{-1} ^{3}HdThd and incubated with shaking at 30°C for 18 h. The labelled bacteria were resuspended in 5 ml TGY broth at 30°C, incubated for 1 h at 30°C, centrifuged and resuspended in 2.5 ml of 67mM phosphate buffer and then UV irradiated. The irradiated bacteria were resuspended in an equal volume of TGY at 30°C and incubated in 0.5 ml samples in microcentrifuge tubes. Excision of dimers was terminated by adding 0.1 ml of calf thymus DNA (5mg ml^{-1}) and 0.6 ml of ice-cold 12.5% trichloroacetic acid (TCA) (Koch Light). Tubes were then held on ice, centrifuged and the precipitate washed with ice-cold 5% TCA and then with ice cold ethanol. The pellets were resuspended in 0.2 ml 98% formic acid and transferred to 1 ml freeze drying ampules which were sealed and heated to 175° for 1 h. The brown hydrolysates were spotted onto Whatman 3MM chromatography paper, dried then run for 18 h in descending chromatography tanks in butanol:acetic acid:water (18:12:30). The paper was dried and cut into 1 cm strips which were placed in scintillation vials containing 0.5 ml water to dissolve labelled nucleotides. 4.5 ml of scintillant (NE250-Nuclear Enterprises, Edinburgh) was added

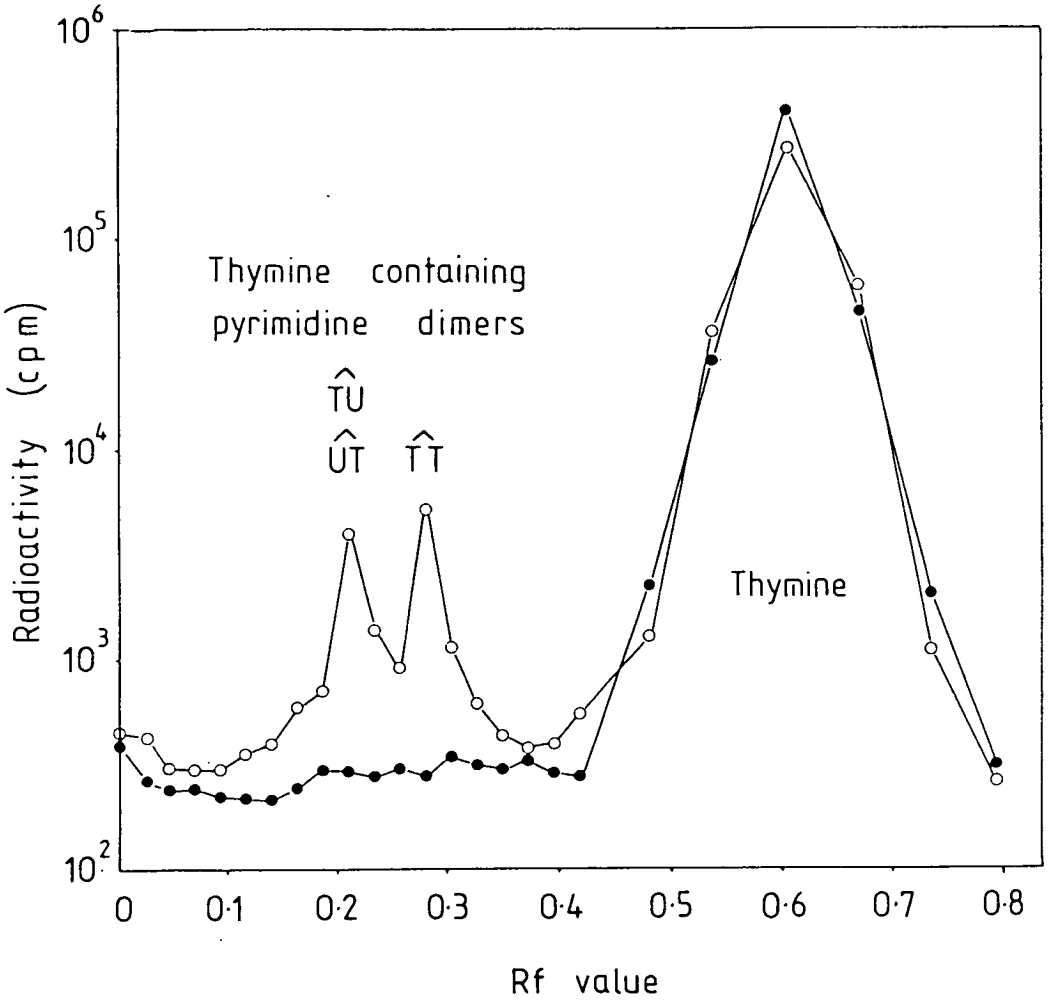


Figure 6

Distribution of radioactivity in paper chromatograms of formic acid hydrolysates of *D.radiodurans* chromosomal DNA labelled with ^{3}H-thymidine before (●) and after (○) UV irradiation (375 Jm^{-2}).

Uracil-thymine (UT) dimers and thymine-uracil (TU) dimers are hydrolysis products of cytosine-thymine and thymine-cytosine dimers respectively (Bollum 1959).

to each vial and the radioactivity assayed in a scintillation counter (Packard Instruments Ltd., Caversham, Bucks). The percentage of thymine present in thymine containing pyrimidine dimers was calculated from the ratio of counts found in the dimer peaks and the thymine peak. The distribution of radioactivity obtained is shown in fig.6. The peaks observed at Rf values of 0.21 and 0.29 were only present when bacteria were UV irradiated and increased in size relative to the major peak at Rf 0.62 in proportion to the UV dose indicating that they are UV photoproducts. The Rf values of 0.21 and 0.29 correspond with the expected positions of uracil-thymine and thymine-thymine dimers respectively as previously determined for this solvent system (Patrick and Rahn, 1976).

Alkaline sucrose gradients

Alkaline sucrose gradients (4.5 ml) were constructed in 5 ml polypropylene centrifuge tubes (MSE). A shelf of 0.2 ml of 40% sucrose dissolved in 0.7M NaCl, 0.3M NaOH, 1mM EDTA (NNE) was placed in the bottom of the tube. A linear 5-20% sucrose gradient in NNE was constructed on top of this using a gradient maker. The gradient was pumped into the tube using a Pharmacia P3 peristaltic pump attached to a capillary. A small glass rod which was the same height as the tube was glued to the end of the capillary and rested against the tube wall. The gradient therefore emerged from the capillary at the top of the tube and ran down the tube between the glass rod and the centrifuge tube wall as a smooth flow. The capillary-glass rod combination was housed in a pasteur pipette which held the glass rod steady and which allowed the capillary-

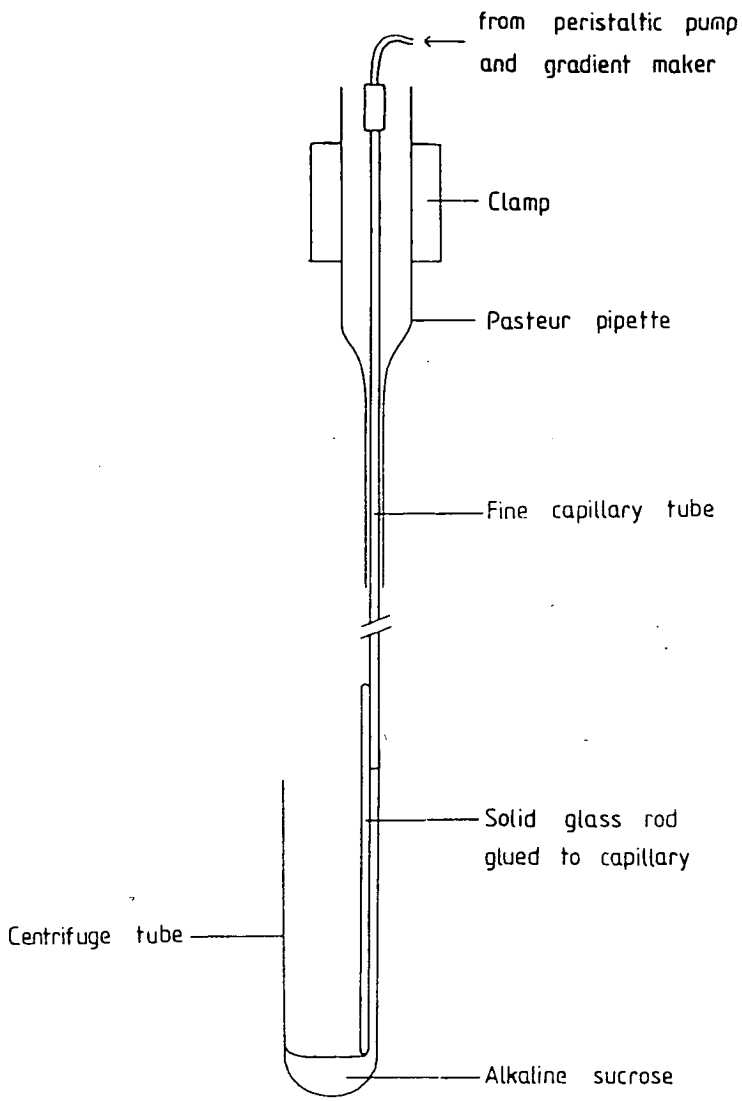


Figure 7

Apparatus used for loading alkaline sucrose gradients into centrifuge tubes.

glass rod combination to be withdrawn with minimal disturbance to the gradient. The gradient was overlaid with 0.2 ml 0.5M NaOH using the same device (Fig.7).

Preparation of cells for alkaline sucrose gradients

Bacterial DNA was labelled by growing bacteria for 18 h in TGY containing ^{3}HdThd (20μCi ml^{-1}). Exponentially-growing bacteria were resuspended twice in 67mM phosphate buffer pH 7.0 prior to UV irradiation or in the appropriate medium if bacteria were exposed to DNA-damaging chemicals. The phosphate buffer also contained 20mM EDTA or 20μg ml^{-1} chloramphenicol in some instances. After exposure to DNA-damaging agents samples were withdrawn, centrifuged and resuspended in ice cold BSPEB (0.5 ml). Some samples were incubated in TGY or TGY containing chloramphenicol (15μg ml^{-1}) for various times prior to resuspension in BSPEB buffer. Cells in BSPEB were held on ice for 4 min then resuspended in PEB buffer containing lysozyme (0.5mg ml^{-1}) and incubated at 37°C for 25 min. About 0.1 ml of this suspension containing approx 10^{7} bacteria was layered carefully onto the top of the alkaline sucrose gradient using a Finnpipette with the first 1 cm of the 'plastic tip' cut off.

Analysing gradients

Gradients were centrifuged for 105 min at 30K rpm in an MSE 6x5 ml swing out rotor at 18°C. Fractions were collected from the gradient by careful insertion of a fine capillary through the centre of the gradient to the bottom of the tube through a pasteur pipette which held the capillary steady. The gradient was pumped out through the capillary using a

peristaltic pump and dripped onto Whatman 3MM chromatography paper strips marked at 1 inch intervals, approximately 0.1 ml (7 drops) was dripped onto each 1 inch segment. Each strip was dried and washed in two changes of cold 5% trichloroacetic acid for 20 min and then in two changes of absolute ethanol. Strips were dried, cut up and each piece placed in a scintillation vial with 10 ml of a toluene-based scintillant (NE233 Nuclear Enterprises). Radioactivity was determined in a scintillation counter. The linearity of gradients was determined by measuring the refractive index of fractions, collected from sample gradients, in a refractometer (Korba *et al.*, 1981) (Fig. 8).

Calculation of the number of single strand breaks in DNA

To calculate the molecular weight of *D.radiodurans* DNA in any particular alkaline sucrose gradient fraction, the gradients are calibrated using DNA of known molecular weight (T4 and λ DNA). The distance that this DNA travels in the gradients allows calculations of the constant β in the formula

$$\beta = \frac{SD}{rpm^2 h} \qquad \text{equation (1) Studier (1963)}$$

where S = sedimentation coefficient of the DNA, D = distance the DNA travels in the gradient (ie which fraction the DNA is found in), rpm = rotor speed, h = centrifugation time. When β is known, substitution of the distance travelled by DNA of unknown MW into the same formula yields a value of S which is related directly to MW by the empirical equation of Studier (1963) and Korba *et al.*, (1981).

$$S = 0.0528\ MW^{0.41} \qquad \text{equation (2)}$$

Values of β obtained in this way using one DNA MW marker are only correct if an arbitrary estimate is made of how far down the centrifuge tube the effective gradient starts. Inclusion of the top gradient fractions leads to spurious β values since they are not part of the gradient proper. The distance from the top of the liquid in the tube to the beginning of the effective gradient can be determined by using two MW markers since the distance they travel from the top of the effective gradient is different by a theoretical constant factor (k), calculated from equations (1) and (2) which for T4 and λ is 1.66 (ie $D_{T4} = 1.66 D_{\lambda}$). By comparing the measured distances that T4 and λ DNA sediment in these gradients (Fig.8) using increasing distances from the top of the gradient as the start point of the measurement, the value of k changes. If the top five fractions of the gradient are ignored in calculations k = 1.63 which is the closest to the theoretical value obtainable.

Now that the true start point of the gradient is known (ie five fractions down the tube) the value of β can be calculated from the distance sedimented by T4 and λ DNA using equations (1) and (2). This gives β values of 9.54×10^9 from T4 and 9.84×10^9 from λ. These are averaged here to give a value of 9.69×10^9 that is used to calculate the MW that corresponds to each gradient fraction, again from equation (1) and (2), which when combined and rearranged for the conditions in these gradients is

$$(116.522D)^{\frac{1}{0.41}} = MW$$

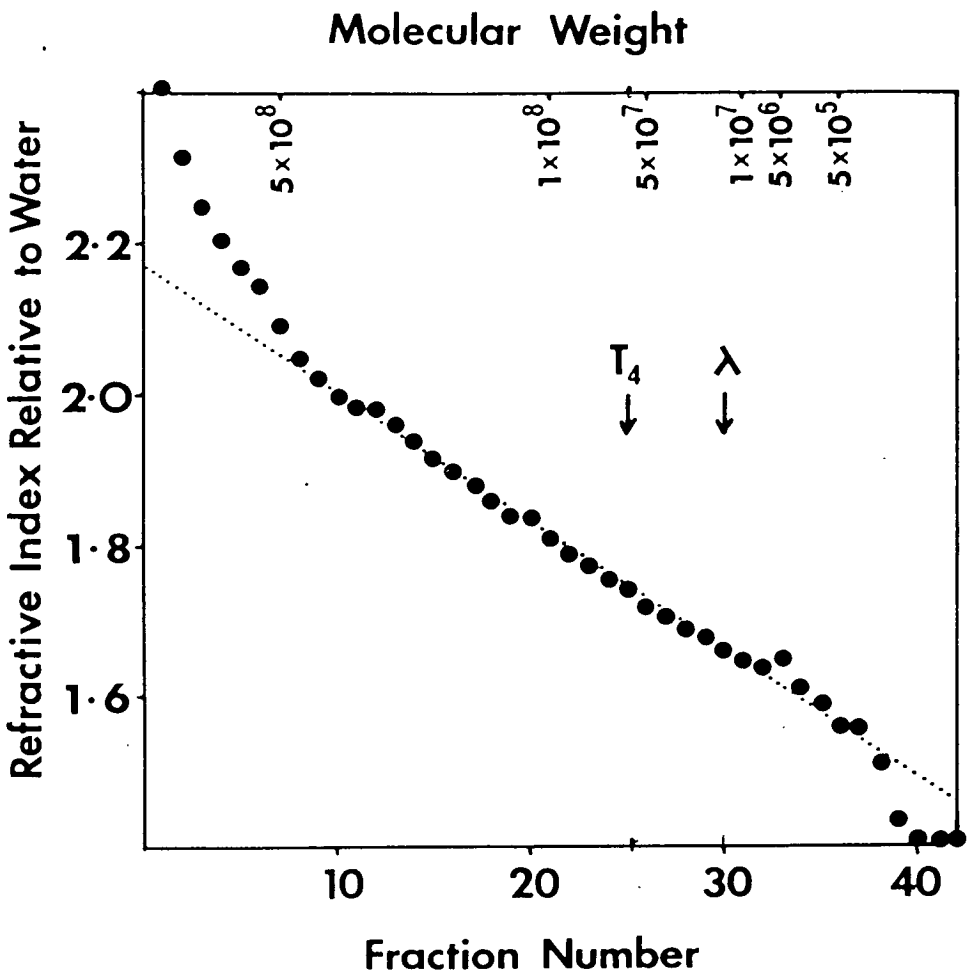


Figure 8

The relative refractive index of fractions obtained from alkaline 5-20% sucrose gradients and molecular weight calibration of gradients using T4 and λ DNA as standard molecular weight markers.

where D = fraction number from the top of the effective gradient.

The DNA from the bacteria sediments over a range of fractions and a measure of average MW must be obtained either as the "weight average molecular weight" (M_w) or the "number average molecular weight" (M_n) (Lett, 1981). M_n is required for estimation of the number of strand breaks in bacterial DNA but is sensitive to errors introduced by the inherent difficulties of measuring accurate molecular weights and distances in the upper part of the gradients (Lett, *et al.*, 1971). Lett *et al.* (1970) suggest that M_w should be calculated which is less sensitive to errors and M_n then obtained from $M_w = 2M_n$ which should apply for a random size distribution of DNA. However this relationship was found not to hold for heavier distributions of DNA which should be nearest to $M_w = 2M_n$ and so M_n was calculated directly from the equation.

$$M_n = \frac{\Sigma Wi}{\Sigma Wi/Mi}$$

where W_i = the weight of DNA in the ith fraction (ie the % of total radioactivity in the ith fraction which is proportional to the weight of DNA) and M_i = the molecular weight which corresponds to the ith gradient fraction.

The number of single strand breaks is expressed as the number per genome, which is 2.0×10^9 daltons (Hansen, 1978).

DNA degradation

Overnight cultures were diluted into TGY medium containing 20μCi ^{3}HdThd and grown for 18 h at 30°C. Exponentially growing

bacteria were resuspended in fresh TGY and allowed to grow for a further 1 h to deplete intracellular pools of label. To confirm this bacteria were treated with BSPEB and PEB containing lysozyme as described previously, lysed with SDS (1%) and the cell contents precipitated with ice cold 12.5% TCA. Less than 1% of the radioactivity was found in the neutralised TCA soluble precipitate. Cultures were UV irradiated in 67 mM phosphate buffer and resuspended in TGY broth (in some instances containing chloramphenicol). Two 20µl and one 100µl samples were withdrawn at various times during incubation at 30°C in an orbital shaker. The 100µl samples were placed in microcentrifuge tubes containing 50µl of a concentrated suspension of unlabelled *D.radiodurans* in 67 mM phosphate buffer pH 7.0 which ensured complete sedimentation of the labelled cells upon centrifugation. 100µl of the supernatant was removed and the radioactivity determined using dioxan-based scintillant (NE250). The two 20µl samples were placed on 1.8 cm Whatman no.1 filter discs which were dried, washed twice in 5% TCA, twice in absolute ethanol, dried, and the radioactivity determined using toluene based scintillant (NE233). The amount of radioactivity measured on the discs was corrected for losses and differences in counts obtained with the different scintillants. Label released into the medium was expressed as a percentage of that on the discs at zero time. DNA degraded is shown as the amount of label released into the medium.

Measurement of DNA synthesis

Overnight cultures of *D.radiodurans* were diluted and grown to a nephelometer reading of 30, resuspended in the same volume of 67 mM phosphate buffer and UV irradiated for various lengths of time. 20µl samples were withdrawn and added to 1/4 oz. vials containing 170µl of TGY medium and 10µCi of ^{3}H-dThd. The vials were incubated in an orbital shaker at 30°C. 10µl samples were withdrawn from the vials at various times and placed on 1.8 cm filter discs (Whatman no.1) which rested on aluminium foil. When the culture had been absorbed by the paper, the discs were placed in two changes of ice cold 5% TCA for 30 min and then two changes of absolute alcohol for 30 min. The discs were dried, placed in scintillation vials with 5 ml of scintillation fluid (NE233) and the radioactivity determined in a scintillation counter.

Preparation of plasmid DNA

Plasmid DNA was prepared essentially according to the method of Ish-Horowicz and Burke (1981). *E.coli* HB101 containing the appropriate plasmid was grown in LBP broth, supplemented with kanamycin (10µg ml^{-1}) in the case of the strain carrying the pML2 plasmid and ampicillin (10µg ml^{-1}) in the case of the strain carrying pAT153. One litre of medium was inoculated with 10 ml of a stationary-phase culture and grown to an optical density of 60 (approximately 10^{9} vu ml^{-1}) at which point chloramphenicol (0.1 g) was added and the culture shaken for 18 h at 30°C to amplify the copy number of the plasmid. The bacteria were harvested by centrifugation at 7000 g for 5 min

and the pellet resuspended in 36 ml of buffer containing 25 mM Tris HCl pH 8.0, 10 mM EDTA and 50 mM glucose. Four ml of the same buffer containing lysozyme (20 mg ml^{-1}) was added and the suspension allowed to stand for 10 min at room temperature. The cells were lysed by adding 80 ml of a solution containing 0.2M NaOH, and 1% SDS and gently inverting the 250 ml sealed plastic centrifuge tube. Unwanted chromosomal DNA and protein was precipitated by the addition of 40 ml of ice-cold potassium acetate pH 4.8 and slow continuing inversion of the sealed tube for 15 min at 4°C. The precipitate was removed by centrifugation at 12000 g for 15 min at 0°C and the supernatant poured into a measuring cylinder through a plastic tea-strainer to remove loose strands of precipitate. AnalaR isopropanol (BDH, 0.6 volumes) was added and the resulting cloudy solution stored at -20°C for one hour before the plasmid was collected by centrifugation at 10,000 g for 10 min at 0°C. The supernatant was discarded and the precipitate resuspended gently in 5 ml 10 mM Tris HCl pH 7.5. The concentration of DNA at this point was typically 1.0 mg ml^{-1} measured by its absorption at 260 nm. This plasmid preparation was stored at 4°C as a source of crude plasmid for further purification. Plasmid DNA was further purified by buoyant density centrifugation in caesium chloride gradients. 4.5 ml gradients were constructed containing 1 mg ml^{-1} of ethidium bromide. The refractive index of gradients was 9.3 measured in a refractometer (Bellingham Stanley Ltd., England). Gradients were overlain with 200µl of liquid paraffin and centrifuged in sealed polypropylene tubes (MSE) at 45000 rpm in a 10x10 ml angle rotor (MSE) for 60 h. Two bands were visible after

centrifugation the upper being chromosomal and linear plasmid DNA and the lower supercoiled covalently closed circular plasmid DNA (ccc DNA). The lower band was removed by piercing the side of the centrifuge tube with a wide bore syringe needle and extracting the relevant band. The visualisation of bands was enhanced at this stage by illumination of the tube with 302 nm light provided by a UV transilluminator (UV Products, Cambridge). Unwanted wavelengths which promote spontaneous breakage of ccc DNA (<302 nm) were excluded by a piece of clear perspex placed in front of the transilluminator. The volume of the extracted liquid was under 0.75 ml. Ethidium bromide was removed from the plasmid by repeated extraction against isopropanol which was saturated with NaCl saturated distilled water. CsCl was removed by the addition of two volumes of distilled water, 1/10 volume of 4M sodium acetate and 6 volumes of ice-cold absolute alcohol which precipitates the plasmid DNA but not the CsCl. The plasmid DNA was pelleted after incubation at -20°C for 1 h by centrifugation in a microcentrifuge for 5 min at 4°C. The pellet was retained and as much of the supernatant as possible removed before resuspension of the plasmid in 10 mM Tris HCl pH 7.5. This was stored as stock and was diluted before each experiment to yield a concentration of plasmid DNA of approximately 200μg ml^{-1}. This method yields approximately 3 mg of supercoiled DNA from one litre of culture.

Preparation of PM2 DNA

Two litres of Q medium in two flasks was inoculated with 40 ml of an overnight culture of *Alteromonas espejiana* BAL31

grown at 20°C. The cultures were shaken at 20°C until the OD reached 60 and 100 ml of fresh PM2 lysate added to each flask. Fresh lysate was prepared by adding an equal volume of stock PM2 lysate in Q medium to an overnight culture of *A.espejiana*. After incubation at 20°C for 1½ h on an orbital shaker the culture lysed, providing sufficient phage to lyse the large culture. After 1 to 2 h the large culture lysed. The phage was precipitated by adding 96 gl^{-1} of polyethylene glycol 8000 and 5 gl^{-1} of NaCl, the additives were dissolved by continuing the shaking at 20°C for 20 min. The cultures were left overnight at 4°C. The precipitated phage was collected by centrifugation at 7000 g for 10 min and resuspended in 20 ml of the supernatant. The phage was again pelleted in a single 50 ml centrifuge tube by centrifuging (10000 g for 10 min). The supernatant was discarded and 30 ml of 1M NaCl in TE buffer added to the pellet. The phage pellet was resuspended and the cell debris removed by centrifugation at 10000 g for 10 min. The pellet was extracted with 1M NaCl in TE buffer and centrifuged; both this and the previous supernatant were pooled and spun at 20000 g at 4°C for 3 h to pellet the phage. The phage was left overnight covered in 3 ml 1M NaCl in TE buffer before being resuspended. The suspension was dialysed against 0.1M NaCl in TE buffer overnight. The DNA was extracted by adding an equal volume of fresh phenol saturated with 0.1M NaCl in TE buffer. The upper aqueous phase was removed and extracted with phenol again and the two aqueous phases combined and dialysed extensively against TE buffer. Approximately 1 mg ml^{-1} (5 ml) of DNA was obtained. DNA was further purified in CsCl gradients as for plasmid DNA.

Damaging DNA

UV irradiation. 50µl quantities of plasmid DNA (200µg ml^{-1}) were placed on a petri dish and irradiated with 254 nm UV light at a dose rate of 1.05 $Jm^{-2}sec^{-1}$.

Introduction of apurinic sites. Stock DNA was precipitated by addition of 0.1 volume of 4M sodium acetate and 0.6 volumes of isopropanol. The DNA was pelleted by centrifugation in a microcentrifuge for 5 min at 4°C and redissolved in buffer containing 0.1M NaCl and 0.1M sodium acetate pH 4.5. The DNA was heated to 70°C for 15 min, placed on ice and the pH raised to neutrality by the addition of 0.1 volume of 0.2M Tris HCl pH 8.0. This method introduces approximately 2 to 3 sites per DNA molecule of this size (Teebor, 1981). DNA was used within 1 week of preparation.

Treatment with EMS or MMS. EMS or MMS was added to DNA in 10 mM Tris HCl pH 7.5 to a final concentration of 125 mM or 10 mM respectively and left at room temperature for 30 min. The DNA was precipitated by the addition of 0.1 volume of 4M sodium acetate and 0.6 volumes of isopropanol, resuspended in 10 mM Tris HCl pH 7.5 and dialysed overnight against two litres of the same buffer. This was sufficient to introduce more than one alkylation specific endonuclease site into each plasmid molecule.

Damaging DNA with Osmium Tetroxide

500 µl of ccc PML2 DNA was made 0.02% with respect to OsO_4 by addition of stock 4% OsO_4 in distilled water. The DNA was then heated to 70°C for 5 min then cooled on ice. OsO_4 was removed by five extractions with water saturated

diethyl ether (BDH, AnalaR) and then the DNA precipitated as described in other sections and resuspended in 10 mM Tris HCl pH 7.0. This procedure introduced at least one pyrimidine hydrate per plasmid molecule and probably not more than 3-5 (Katcher and Wallace, 1983).

Treatment with 8-methoxypsoralen plus near UV light

8 Methoxylpsoralen (MOP) was added to plasmid DNA to a final concentration of 4 μg ml^{-1}. 50μl quantities of DNA (200 μg ml^{-1}) were then irradiated on a petri dish with 365 nm light from a Philips black light (2x40 watt tubes) at a distance of 20 cm.

Production of single-stranded heat-denatured DNA. Double stranded DNA was heat denatured in TE buffer by sealing it in a 1 ml freeze drying ampule, heating it to 100°C for 15 min and then cooling it quickly on ice for 10 min.

Preparation of extracts for assay of UV endonuclease activity

Three litres of cells were grown overnight in TGY medium to stationary phase, centrifuged, washed and resuspended in ice cold buffer (20 ml) containing 50mM Tris HCl pH 7.5 0.1M NaCl 10mM mercaptoethanol and 0.1mM phenylmethylsulphonyl-fluoride (PMSF - a serine protease inhibitor). The cells were broken in a French pressure cell at 40000 p.s.i. at 0°C. Extracts were kept at 0-4°C from this point. The crude lysate was spun at 25000 g for 10 min and the clear red supernatant collected and is the 'crude lysate' referred to in the text which was used for some assays of UV endonuclease activity. The 45-65% ammonium sulphate cut (Fraction A) used in other assays and for the

further purification of UV endonuclease β and the HUV endonuclease was obtained by slowly adding an equal volume of an ice-cold, degassed, 90% saturated ammonium sulphate solution (Sigma Grade 1, purified, 660 g l^{-1}, pH 7.5 containing PMSF (0.1 mM) to crude lysate over 15 min using a peristaltic pump and a magnetic stirrer. (Adding solid ammonium sulphate reduces the UV endonuclease β yield). The resulting solution was stirred for a further 30 min. The precipitate which formed was removed by centrifugation (12000 g for 10 min) which produced a solid bright red pellet consisting of 50-68% of the soluble protein in the lysate. The pellet is discarded and an equal volume of 90% saturated ammonium sulphate added to the clear, off-white supernatant in the same way as previously described to give 65% saturation. A white pellet was obtained after centrifugation (12000 g for 10 min) which contained 10-15% of the soluble protein. The pellet was gently redissolved in 2 to 3 ml of 10 mM Tris HCl pH 7.0, 0.1 mM PMSF over 30 min. The resulting solution was dialysed against 4 l. of the same buffer for 18 h and its protein content assayed. This is Fraction A which was divided and stored at -70°C in microcentrifuge tubes until required.

Detection of endonuclease activity

The detection of endonuclease activity relies on the different electrophoretic mobilities of supercoiled covalently closed circular (ccc) DNA and open circular (oc) DNA. Conversion of ccc DNA to oc DNA requires only one DNA single-strand break per ccc molecule. ie. each molecule requires only one endonuclease incision for the conversion. The two

forms of DNA are identified qualitatively after separation in agarose gels. Damage-specific endonuclease activity converts damaged ccc DNA but not undamaged ccc DNA to oc DNA. Each assay tube contained 10 µl of extract to be assayed, 5 µl of damaged or undamaged ccc DNA (approx 1 µg) and 5 µl of any addition at four fold greater concentration than required. Assay mixtures were typically incubated at 30°C for 30 min and reactions were halted by the addition of 20 µl of STOP buffer. The whole assay mixture was loaded into one well of an agarose gel.

Agarose gel electrophoresis of DNA

Agarose (0.8% or 1%, low EEO Agarose, Sigma) was boiled in TAE buffer, cooled to 50°C and then cast in a home made apparatus. The gel dimensions were 120x5x80 mm. When set, samples of DNA were added to wells in the gel and TAE buffer added up to the level of the gel surface. The apparatus was constructed so that the only circuit was directly through the gel and allowed large currents to be obtained for very low voltages. This allowed several gels to be run in parallel off one low voltage power pack. A constant 20 volts was typically applied which produced 75 mA per gel. The marker dye in the STOP buffer (bromophenol blue) reached the end of the gel in 2 to 2.5 h.

Visualization of DNA in gels

Agarose or polyacrylamide gels containing DNA were stained with ethidium bromide (10 µg ml^{-1}) for 30 min, destained in water for 30 min and DNA visualized by placing the gels on the 302 nm UV transilluminator.

SDS polyacrylamide gel electrophoresis

Polyacrylamide gels were cast in home-made cassettes made from 4 mm window pane glass and perspex spacers and sealed with paraffin wax. Gel dimensions were approximately 110x150x1 mm and were linear gradients (5-15% or 7-15%) of acrylamide (BDH, specially purified for electrophoresis). Gels contained in addition a linear gradient of glycerol (0-10%) running in the same direction as the acrylamide gradient. The gradients were constructed in a home-made gradient maker and were pumped into the cassette over a period of 15 min. The ratio of acrylamide to bisacrylamide in the gels was 36.5:1. The main resolving gradient gel contained in addition to acrylamide 0.1% SDS (BDH, specially purified) and 0.375M Tris HCl pH 8.8. Polymerisation was initiated by the addition of 0.1% tetramethylethylenediamine (TEMED, BRL Chemicals, high purity) and 0.12% ammonium persulphate and was complete within 1 h. In some instances the resolving gel also contained salmon sperm DNA (10.20 $\mu g\ ml^{-1}$) that was either native or heat-denatured Water-saturated n-butyl alcohol was added to the gels while they were setting to create an even surface for contact with the stacking gel and to prevent the gel drying out. The gels were left overnight at 4°C. After removal of the n-butyl alcohol the resolving gel was overlaid with a 5 ml stacking gel just prior to use which was cast containing the slots for protein extracts. The stacking gel consisted of 0.125M Tris HCl, pH 6.8, 0.1% SDS, 0.3% TEMED, 0.3% ammonium sulphate and 4% acrylamide and was set within 20 min. Lanes were loaded with 20-50 µg of protein and the gel was run at a constant current of 20mA in a vertical home-made tank apparatus. The electrode

buffer consisted of 0.2M glycine, 0.01% SDS and 0.05M Tris base. Protein separation took approx 5 h and total proteins were then visualized either with silver staining by the method of Sammons (1981) or by incubating the gel overnight in a solution containing 0.25% w/v kenacid blue R250 (BDH), 45% methanol and 8% glacial acetic acid and then for 24 h in a solution containing 5% methanol and 7.5% glacial acetic acid. Alternatively, nucleases were visualized in DNA containing gels (Rosenthal and Lacks, 1977) by removing the SDS and glycerol from the gels by washing them with several changes of 10mM Tris HCl pH 7.0. The gels were then cut into individual lanes in some instances and incubated at 37°C for 3 to 7 days in the same buffer in some instances containing divalent cations (5mM) or ATP (5mM) or mercaptoethanol (5mM) or NaCl (25mM) in various combinations. Bacterial growth was suppressed by addition of 0.01% sodium azide to the incubation buffers.

Preparation of proteins for polyacrylamide gel electrophoresis

Whole cell lysates were prepared by resuspending 20 ml of exponentially-growing bacteria (OD 40) in ice-cold BSPEB (1 ml) for 4 min, then in PEB containing lysozyme (0.5 mg ml^{-1}) for 25 min at 37°C, and then 0.5 ml of a solution containing 0.125M Tris HCl pH 6.8, 2% SDS, 10% glycerol and 0.005% (w/v) bromophenol blue (lysis buffer) was added to lyse the cells. 50 µl of the final solution was loaded onto the gel (approx 50 µg protein). Other proteins or extracts were mixed 1:1 with lysis buffer and loaded directly onto the gel.

Assay of protein concentration

Protein concentration was assayed using a Bio-Rad (Watford, Herts) protein assay kit.

Photography

Photographs were taken using a Polaroid MP4 camera system with a 35 mm lens on black and white 5x4 inch positive-negative instant prints. Gels stained with silver or kenacid blue were photographed on a white light box for ½ sec at f11. Gels stained with ethidium bromide were photographed through a kodak wratten no. 9 filter which cuts out UV glare less than 300 nm wavelength and a red filter obtained from the journal Nature (vol. 284. no.5755, 1980) which cuts out wavelengths between 400-600 nm. Exposure was for approx 10 min at f4.5.

Preparation of chromatography materials

DEAE sephacel (Pharmacia, Milton Keynes) was resuspended several times in an excess of 50 mM Tris HCl pH 7.0 and degassed before use. After loading into the column apparatus the matrix was washed with 300 ml 0.1M Tris HCl at the appropriate pH, then with 500 ml of 10mM Tris HCl at the appropriate pH, containing in some instances 1mM EDTA. All solutions were degassed before use. The flow rate during washing was 20 ml h^{-1}. For testing of enzyme binding at various pH values and NaCl concentrations, DEAE sephacel (0.5 ml) was resuspended five times in 0.5M Tris HCl at the appropriate pH, in some cases containing NaCl at the appropriate molarity, and then five times in similar buffer which was 10mM with respect to Tris HCl. Fraction A (approx 25µg) was added to the final supernatant

at 0°C and the matrix held at 0°C for 30 min with occasional remixes. Enzyme activity was assayed in the supernatant at the end of this period. Hydroxylapatite (HT grade, Bio-rad) was prepared in a similar manner to DEAE sephacel except that the buffer was potassium phosphate. Ultrogel ACA 54 (LKB, South Croydon, Surrey) was also washed before use in three volumes of 10 mM Tris HCl pH 7.0, 0.2M NaCl.

All chromatographic procedures, including packing columns were carried out at 4°C.

Preparation of *M.luteus* pyrimidine dimer UV glycosylase

1 litre of TGY medium was inoculated with *M.luteus* and grown overnight to stationary phase. The bacteria were centrifuged at 10000 g for 10 min and resuspended in 15 ml of ice cold 10mM Tris HCl pH 7.5, 5mM EDTA and lysed in a French Press at 30000 lb per sq in. Cell debris was removed by centrifugation at 30000 g for 20 min at 2°C and the supernatant retained. Ice cold 90% saturated $(NH_4)_2SO_4$ was added to 65% saturation over 15 min and stirring continued for a further 30 min at 4°C. The precipitate was collected by centrifugation (10000g for 10 min), redissolved in 3 ml of 10mM Tris HCl pH 7.5, 5mM EDTA, dialysed against 4 litres of the same buffer overnight then frozen at 70°C. Upon thawing, the UV endonuclease activity was assayed or used as for *D.radiodurans* UV endonucleases except that the reaction buffer contained 10mM Tris HCl pH 7.5, 0.1M NaCl and 2mM EDTA. No non-specific incision or degradation of plasmid DNA was observed under these reaction conditions.

CHAPTER 3

Results

Chapter 3

Section 1: Isolation and characterization of DNA repair-deficient mutants

Part A Isolation of DNA-repair-deficient strains

An attempt was made to isolate strains of *D.radiodurans* deficient in excision repair. Although *D.radiodurans* wild-type strains are mutable by MNNG, strain 302 is hypermutable (Tempest and Moseley, 1980) enabling the isolation of larger numbers of DNA repair-deficient strains to be made in order to screen them for a deficiency in excision repair. Strain 302 is UV resistant, MMS resistant and sensitive to mitomycin C. Mutation of strain 302 by MNNG yielded 40 strains which were sensitive to UV irradiation. *E.coli uvr*$^-$ strains are UV sensitive and MMS resistant (Murray 1979) and so strains of *D.radiodurans* were selected from the 40 UV-sensitive strains which remained MMS resistant. Exponentially-growing cells were exposed to 0.5% MMS in phosphate buffer pH 7.0 for 50 min (approx. 50mMh) and the number of surviving cells determined. The D_{37} for the wild-type strain and strain 302 is approximately 80mMh. Twenty five of the UV-sensitive strains were sensitive to this MMS dose with less than 10^{-6} survivors. Survival approached 100% in the remaining 15 strains.

Excision of pyrimidine dimers

The number of pyrimidine dimers induced in the DNA of the wild type *D.radiodurans* strain at various UV doses was investigated in order to select a UV dose that produced sufficient pyrimidine dimers to be conveniently monitored by chromatography but which was sublethal to the wild type strain (Figs.6,12). Strains were irradiated with the selected UV dose (375 Jm^{-2}) which converted approx.0.72% of thymine in DNA

to thymine-thymine (TT) dimers and 0.63% to cytosine-thymine (CT) and thymine cytosine dimers (Table 5). The wild type strain excises both TT and CT (TC) dimers at the same rate of 2.5% of the original number per min (Fig.9). This was equivalent to approx.360 dimers per genome per min. Caffeine (2.5 $mgml^{-1}$) present in the post-irradiation medium had no effect on the excision of pyrimidine dimers in the wild type despite reducing survival of the strain when added immediately after UV irradiation and included in the growth medium (Fig.10). However, 20mM EDTA prevented excision of any pyrimidine dimers during 2h after irradiation when present in the post-irradiation medium (data not shown).

The UV-sensitive, MMS-resistant strains all excised less than 10% of TT or CT (TC) dimers during 2h after UV irradiation.

The excision-repair capabilities of the UV-sensitive, MMS-sensitive strains were also examined to determine if the MMS-resistant phenotype was characteristic of this excision repair deficiency. Eight strains were examined including the UV-sensitive strains 301 and 303 (Moseley and Copland, 1978) which were also found to be MMS sensitive. All were able to excise TT and CT (TC) dimers, although in some instances the excision rate was slower than in the wild type strain (Fig.11).

Selection of strains for further study

The UV-survival curves of the UV-sensitive, MMS-resistant strains fell into three groups differing in general sensitivity and the shapes of the curves. The three groups corresponded closely with the survival curves of three strains UVS9, UVS25

Table 5

Quantity of radioactivity appearing as thymine containing pyramidine dimers after UV irradiation and the number of dimers present in the *D.radiodurans* genome.

UV dose (Jm^{-2})	% label in $\hat{T}\hat{T}$ peak	No. $\hat{T}\hat{T}$ dimers per genome	% label in $\hat{C}\hat{T}$ & $\hat{T}\hat{C}$ peaks	No. $\hat{C}\hat{T}$ & $\hat{T}\hat{C}$ dimers per genome	% label in both peaks	Total dimers per genome
125	0·12	1260	0·09	950	0·21	2200
250	0·33	3500	0·25	2600	0·58	6100
375	0·72	7600	0·63	6600	1·35	14250
500	1·12	11800	0·96	10100	2·08	22000

Radioactivity is expressed as a percentage of the total radioactivity in all chromatographic peaks.

The *D.radiodurans* genome size is 2.0×10^9 daltons (1.06×10^6 thymines per genome) (Hansen 1978).

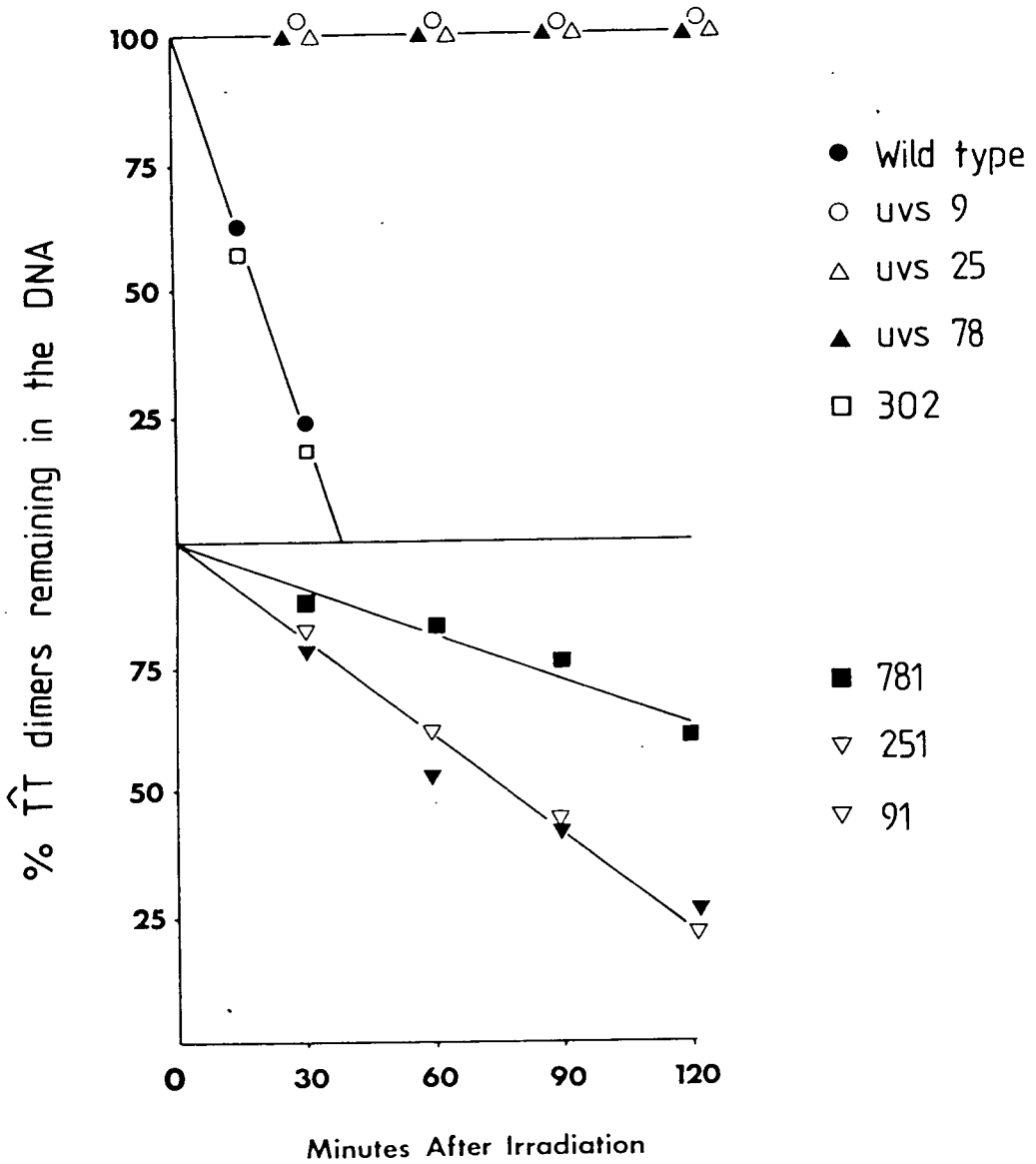


Figure 9

Rate of excision of pyrimidine dimers from the TCA insoluble fraction of the *D.radiodurans* wild type and MMS-resistant, DNA repair-deficient strains after UV irradiation (375 Jm^{-2}). $\hat{TT}$ and $\hat{CT}$ ($\hat{TC}$) dimers are excised at the same rate.

The fraction of pyrimidine dimers remaining TCA insoluble is expressed as a percentage of the numbers of dimers present immediately after irradiation.

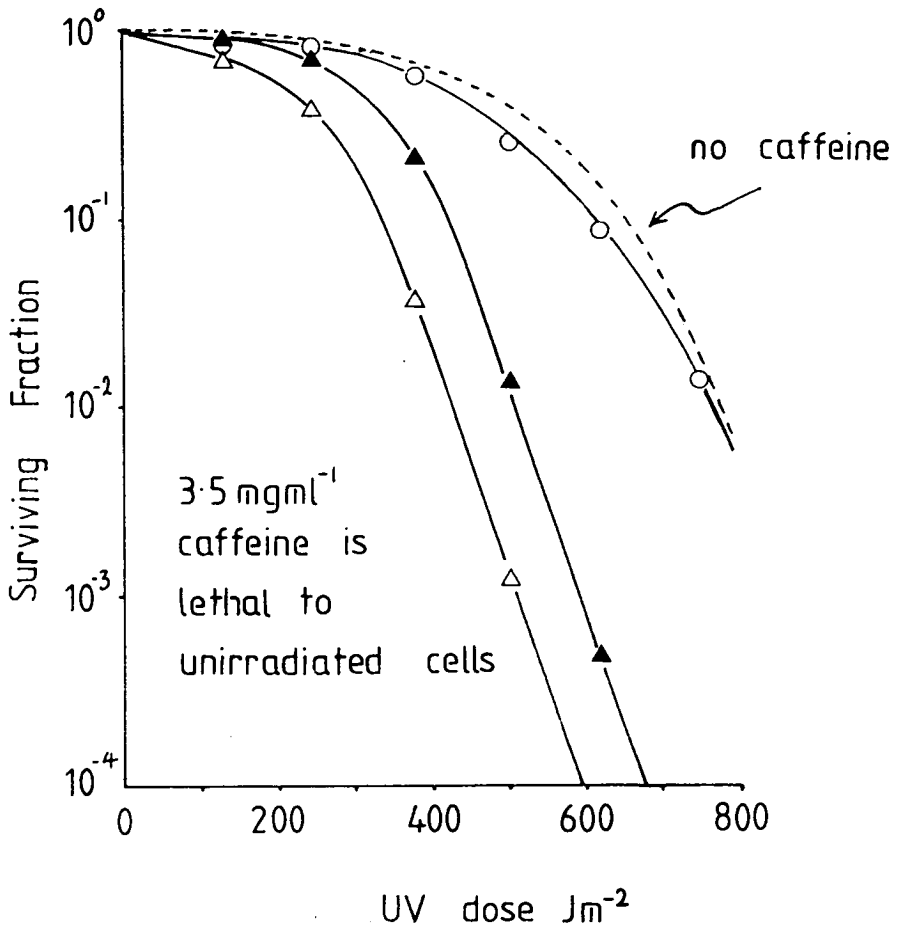


Figure 10

Effect of caffeine on the survival of *D.radiodurans* wild type strain after UV irradiation. Caffeine was added to the bacteria immediately after irradiation and included in serial dilutions and TGY agar.

o 1 $mgml^{-1}$ caffeine

▲ 2.5 $mgml^{-1}$ caffeine

Δ 3.0 $mgml^{-1}$ caffeine

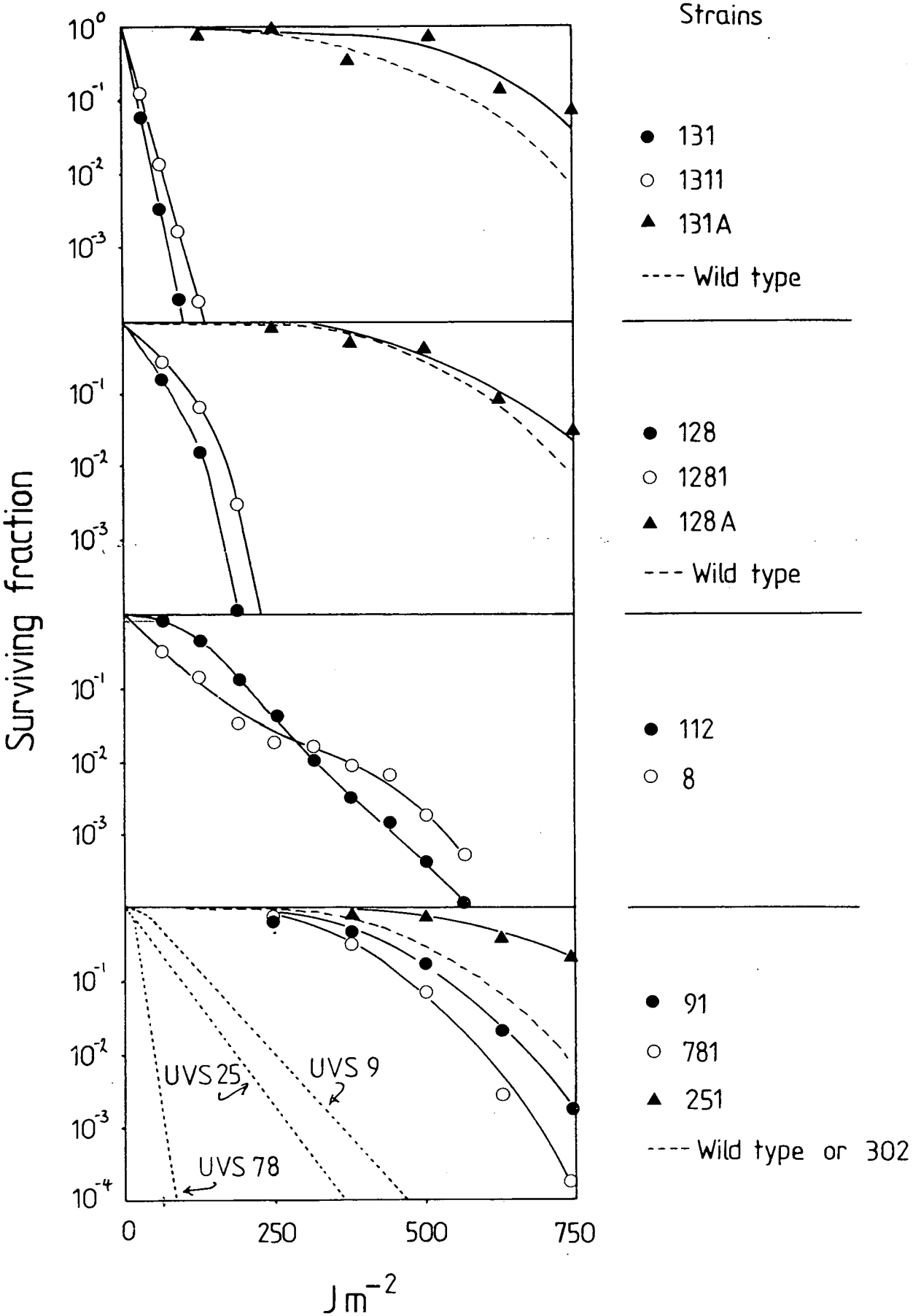
Figure 11

Rate of excision of pyrimidine dimers from the TCA insoluble fraction of MMS-sensitive DNA repair-deficient *D.radiodurans* strains after UV irradiation (375 Jm^{-2}). TT and CT (TC) dimers are excised at the same rate.

The fraction of pyrimidine dimers remaining TCA insoluble is expressed as a percentage of the numbers of dimers present immediately after irradiation.

Figure 12

Survival of DNA repair-deficient *D.radiodurans* strains after UV irradiation.


Strains
131
1311
131A
Wild type
128
1281
128A
Wild type
112
8
91
781
251
Wild type or 302
UVS 25
UVS 9
UVS 78
Surviving fraction
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
0
250
500
750
$J\,m^{-2}$

and UVS78 isolated by Moseley (Fig.12). Strains UVS9, UVS25 and UVS78 were also found to be deficient in excision repair of both TT and CT (TC) dimers (Fig.9). DNA extracted from strains UVS9, UVS25 and UVS78 failed to transform representatives of the corresponding groups to UV resistance but would transform strains 301 and 303 to UV resistance. It was concluded that no additional phenotypes had been isolated over and above the three represented in strains UVS9, UVS25 and UVS78 and therefore these strains were used in subsequent studies.

The UV-survival curves of the MMS-sensitive strains were more varied than those of the MMS resistant strains and so four strains were arbitrarily chosen. Strain 131 was selected because it was the most UV sensitive. Strains 112 and 8 were the most UV-resistant of the group but differed both in their UV survival characteristics and the rate at which they excised pyrimidine dimers. Strain 128 had intermediate UV sensitivity between those of strain 131 and strains 112 and 8. The UV-survival curves of these strains are shown in figure 12.

Removal of the 302 mutation from MMS-resistant strains

In order to study the effects in isolation of the mutations which confer UV-sensitivity on strain 302 the original mutation (*mtcA*) had to be removed. The 302 mutation renders cells sensitive to mitomycin C and can be removed by transformation with DNA containing a wild type *mtcA* gene with selection on agar for mitomycin C resistance ($0.05\mu gml^{-1}$) (Moseley and Copland, 1978). Mitomycin C-resistant clones were isolated from strains UVS9, UVS25, UVS78, 131 and 128 but not from

strains 112 or 8 (Table 6). Mitomycin C-resistant strains obtained from strains UVS9, UVS25, UVS78, 131 and 128 were labelled 91, 251, 781, 131A and 128A respectively. Further attempts to isolate mitomycin C-resistant transformants or even spontaneous mitomycin C resistant mutants of strains 8 and 112 using greater than 10^9 recipient cells consistently failed. No spontaneous mutations to mitomycin C-resistance were obtained from any strain carrying the 302 mutation (Table 7). Transformation of strain 112 also failed to produce rifampicin-resistant clones although they were obtained from strain 8.

Mitomycin C sensitivity, removal of the 302 mutation from MMS-sensitive strains and evidence for linkage

The failure to obtain mitomycin C-resistant transformants of strain 8 prompted examination of the mitomycin C sensitivity of this strain. Relative to the mitomycin C-sensitive parent strain 302, the D_{37} dose for strain 8 was approximately 15 fold lower (Fig.13).

The mitomycin C-sensitivities of other strains mutant in the *mtcA* gene and another DNA repair gene were also compared to strain 302. The MMS-resistant strains UVS9, UVS25 and UVS78 were at least as resistant as strain 302, and in the case of UVS9, slightly more so (Fig.13). The MMS-sensitive strains 112, 128 and 131 were approximately three fold more sensitive to mitomycin C than strain 302. The additional sensitivity of strains 8, 112, 128 and 131 to mitomycin C over that of strain 302 indicated that the second mutation was in a repair pathway distinct from that mediated by the *mtcA* gene and that

Table 6

The frequency of rifampicin-resistant and mitomycin C resistant transformants obtained by transformation of mitomycin C sensitive, DNA repair-deficient *D.radiodurans* strains with DNA from a rifampicin resistant derivative of *D.radiodurans* wild type strain.

	Frequency of resistant transformants	
Strain	Rifampicin (20 $ugml^{-1}$)	Mitomycin C (0·05 $ugml^{-1}$)
UVS 9	$2{\cdot}1 \times 10^{-4}$	$5{\cdot}2 \times 10^{-4}$
UVS 25	$3{\cdot}0 \times 10^{-4}$	$9{\cdot}6 \times 10^{-5}$
UVS 78	$1{\cdot}8 \times 10^{-3}$	$1{\cdot}8 \times 10^{-3}$
131	$2{\cdot}6 \times 10^{-3}$	$2{\cdot}3 \times 10^{-4}$
128	$5{\cdot}5 \times 10^{-4}$	$6{\cdot}6 \times 10^{-5}$
112	$1{\cdot}1 \times 10^{-7}$	$< 1{\cdot}1 \times 10^{-8}$
8	$2{\cdot}6 \times 10^{-3}$	$< 2{\cdot}0 \times 10^{-8}$

Table 7

Spontaneous mutation frequencies to rifampicin resistance, mitomycin C resistance and UV resistance of the *D.radiodurans* wild type and DNA repair-deficient strains.

	Spontaneous mutation frequency		
Strain	Rifampicin (10 $ugml^{-1}$)	Mitomycin C (0·015 $ugml^{-1}$)	UV resistance (300 Jm^{-2})
Wild Type	$1·1 \times 10^{-7}$	N/A	N/A
302	$1·5 \times 10^{-7}$	$< 5·0 \times 10^{-10}$	N/A
UVS 9	$1·4 \times 10^{-7}$	$< 2·7 \times 10^{-8}$	$< 2·5 \times 10^{-7}$
UVS 25	$2·0 \times 10^{-7}$	$< 1·1 \times 10^{-8}$	$< 2·5 \times 10^{-6}$
UVS 78	$5·3 \times 10^{-8}$	$< 5·3 \times 10^{-8}$	$< 4·0 \times 10^{-6}$
131	$1·2 \times 10^{-7}$	$< 2·5 \times 10^{-8}$	$2·5 \times 10^{-7}$
112	$1·4 \times 10^{-7}$	$< 5·0 \times 10^{-10}$	$< 2·8 \times 10^{-6}$
8	$5·0 \times 10^{-7}$	$< 1·0 \times 10^{-8}$	$< 1·8 \times 10^{-7}$
128	$1·7 \times 10^{-7}$	$< 1·8 \times 10^{-8}$	$< 2·2 \times 10^{-7}$

N/A ···· Not applicable

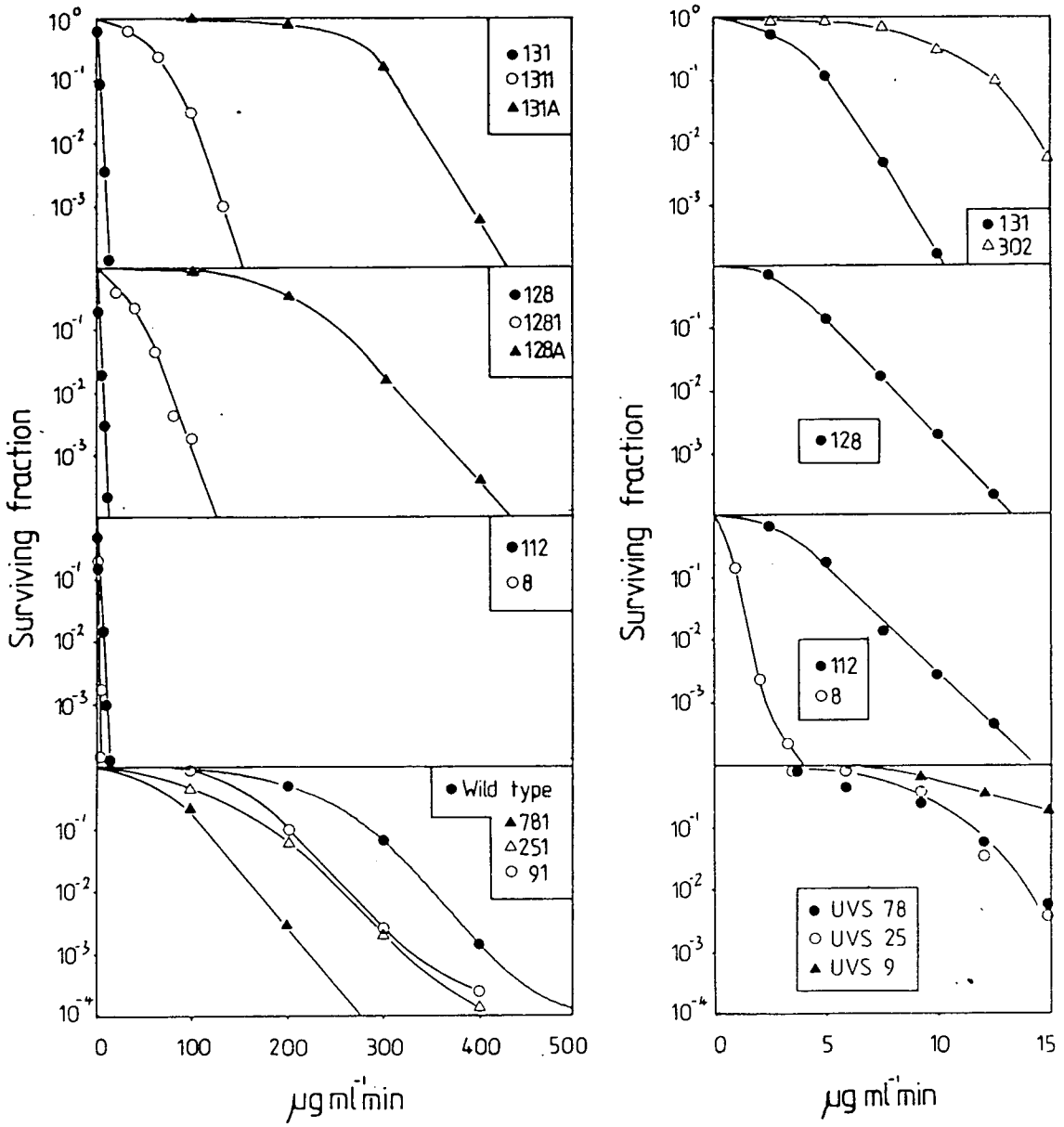


Figure 13

Survival of DNA repair-deficient *D.radiodurans* strains after exposure to mitomycin C.

the second mutation would probably confer a mitomycin C sensitive phenotype in the presence of a functional *mtcA* gene. If this were the case then the isolation of strains 131A and 128A which were as resistant to mitomycin C as the wild type strain was most likely due to double transformation events restoring both the 302 and the 131 or 128 mutations simultaneously. An attempt was therefore made to select derivatives of strains 8, 128 and 131 with mitomycin C sensitivities intermediate between those of the double-mutant strains and the wild type. Strains were transformed with wild type DNA and plated on TGY agar containing the minimum inhibitory concentrations of mitomycin C for these strains, which were $0.005\mu gml^{-1}$ for strain 8 and $0.015\mu gml^{-1}$ for strains 128 and 131. No transformants were obtained from strain 8. Resistant transformants arose at a frequency of $5x10^{-4}$ from strain 128 and $1x10^{-2}$ from strain 131. Approximately 10% of those clones could grow on TGY containing $0.05\mu gml^{-1}$ of mitomycin C when restreaked and were as UV resistant as the wild type strain. The remaining 90% which would not grow on TGY containing $0.05\mu gml^{-1}$ of mitomycin C were sensitive to $250Jm^{-2}$ of UV, ie this dose would kill all bacteria in a streak on a TGY plate. Strains 1281 and 1311 are representatives of the transformants isolated from strains 128 and 131 respectively which grew on $0.015\mu gml^{-1}$ but not on $0.05\mu gml^{-1}$ mitomycin C.

The high proportion of clones arising from transformation of strains 128 and 131 with wild-type DNA, which were resistant to both UV and mitomycin C when resistance to only UV or mitomycin C was selected for, was indicative of linkage between the *mtcA* gene and the additional mutations. The expected

frequency of double transformants of unlinked markers would be expected to be the product of the frequency of single transformants (Hayes 1974). The observed frequency of double transformants relative to the calculated frequency of double transformants increases as the competent fraction decreases for unlinked markers. The competent fraction of *D. radiodurans* populations calculated by the analysis of unlinked markers exceeds 100% and can reach 2300% in an exponentially growing population due to the presence of multiple transformable sites (single strands of DNA in multiple genomes) in each cell (Moseley and Evans 1981). The expected frequency of double transformants must therefore be reduced by a factor corresponding to the number of transformable sites present because the probability of two independent transformation events occurring in the same DNA strand is reduced as the number of genomes per cell increases. The observed frequencies of double transformation events which occurred after initial selection for one marker (UV or mitomycin C resistance) are shown in Table 8. The frequency of double transformants was similar whether UV resistance or mitomycin C resistance was the primary selection. The highest frequency was for strain 128 with 40% of transformants having both resistances restored. The frequency was tenfold lower for strain 131 and none were obtained from strain 301.

High concentrations of donor DNA can cause double transformation events if cells take up more than one DNA strand. This seemed an unlikely source of error here since the same DNA concentration used to produce double transformants of strain 128 failed to produce any from strain 301. However,

Table 8

Frequency of doubly resistant (UV and mitomycin C) transformants obtained after transformation of strains 128, 131 and 301 with DNA obtained from a rifampicin resistant derivative of the *D.radiodurans* wild type strain.

	Frequency of resistant transformants of strains 131, 128 and 301 obtained after transformation with wild type DNA (approx. 500 $\mu g ml^{-1}$)					
	Single transformants			Double transformants		*Expected frequency
Strain	Rifampicin ($10\ \mu g ml^{-1}$)	A UV ($250\ Jm^{-2}$)	B Mitomycin C ($0{\cdot}015\ \mu g ml^{-1}$)	C UV^R clones which are also MTC^R	D MTC^R clones which are also UV^R	$\frac{A \times B}{\text{Genome no.}(5)}$
131	$6{\cdot}7 \times 10^{-3}$	$5{\cdot}7 \times 10^{-3}$	$7{\cdot}7 \times 10^{-3}$	$3{\cdot}7 \times 10^{-4}$	$4{\cdot}1 \times 10^{-4}$	$8{\cdot}8 \times 10^{-6}$
128	$7{\cdot}0 \times 10^{-4}$	$1{\cdot}1 \times 10^{-3}$	$5{\cdot}0 \times 10^{-4}$	$4{\cdot}2 \times 10^{-4}$	$2{\cdot}2 \times 10^{-4}$	$1{\cdot}1 \times 10^{-7}$
301	$4{\cdot}6 \times 10^{-4}$	$4{\cdot}0 \times 10^{-5}$	$2{\cdot}0 \times 10^{-4}$	$< 1{\cdot}0 \times 10^{-7}$	$< 7{\cdot}0 \times 10^{-7}$	$8{\cdot}0 \times 10^{-9}$

	% Linkage		
Strain	$\frac{C}{A} \times 100$	$\frac{D}{B} \times 100$	Average $\frac{C+D}{A+B} \times 100$
131	6·5	5·3	5·8
128	38·1	44·0	40·0
301	< 0·25	< 0·35	< 0·33

UV^R - UV resistant

MTC^R - Mitomycin C resistant

* - Expected frequency of transformants if the two markers were unlinked

to eliminate the possibility strain 131 (which transforms at the highest frequency) was transformed with decreasing concentrations of DNA. Unlinked markers would be expected to cotransform at a 100 fold lower frequency for every 10 fold reduction in donor DNA concentration whereas the frequency of double transformants produced by linked genes reduces at the same rate as the frequency of single transformants. The observed reductions in frequencies are shown in Table 9 and indicate that the double transformation events are due to linkage because; i) the observed frequency of double transformants always exceeds the expected frequency for cotransformation of unlinked markers, ii) the reduction in frequency of double transformants is approx tenfold (and not 100 fold) for a tenfold reduction in donor DNA concentration, iii) although the degree of linkage apparently falls tenfold when the donor DNA concentration is reduced from $100\mu gml^{-1}$ to $10\mu gml^{-1}$ this is not reduced further. The apparent reduction in the degree of linkage could be the result of a component of the double transformation events being unlinked cotransfer of markers but this implies that 90% of the double transformants are due to cotransformation of unlinked markers. This seems doubtful in view of the failure to detect them in strain 301 and it may therefore be due to an experimental artifact.

Uptake of DNA by strain 112

The non-transformability of strain 112 prompted measurement of the ability to take DNA into the cell. Exponentially growing bacteria were incubated with ^{3}H DNA under conditions for transformation then washed and treated with DNase ($50\mu gml^{-1}$ for

Table 9

Frequency of doubly resistant (UV and mitomycin C) transformants obtained after transformation of strain 131 with varying concentrations of DNA obtained from a rifampicin resistant derivative of the *D.radiodurans* wild type strain.

	Frequency of resistant transformants of strain 131 obtained after transformation with wild type DNA					
	Single transformants			Double transformants		* Expected frequency
DNA conc. ($\mu g ml^{-1}$)	Rifampicin ($10\ \mu g ml^{-1}$)	A UV ($250\ jm^{-2}$)	B Mitomycin C ($0{\cdot}015\ \mu g ml^{-1}$)	C UV^R clones which are also MTC^R	D MTC^R clones which are also UV^R	$\frac{A \times B}{\text{Genome no.(5)}}$
1	$3{\cdot}1 \times 10^{-4}$	$3{\cdot}9 \times 10^{-4}$	$4{\cdot}0 \times 10^{-4}$	$2{\cdot}1 \times 10^{-6}$	$2{\cdot}7 \times 10^{-6}$	$3{\cdot}1 \times 10^{-8}$
10	$8{\cdot}0 \times 10^{-4}$	$4{\cdot}6 \times 10^{-3}$	$2{\cdot}0 \times 10^{-3}$	$3{\cdot}1 \times 10^{-5}$	$1{\cdot}3 \times 10^{-5}$	$1{\cdot}8 \times 10^{-6}$
100	$7{\cdot}5 \times 10^{-3}$	$6{\cdot}8 \times 10^{-3}$	$1{\cdot}2 \times 10^{-2}$	$4{\cdot}3 \times 10^{-4}$	$3{\cdot}9 \times 10^{-4}$	$1{\cdot}6 \times 10^{-5}$

	% Linkage		
DNA conc. ($\mu g ml^{-1}$)	$\frac{C}{A} \times 100$	$\frac{D}{B} \times 100$	Average $\frac{C+D}{A+B} \times 100$
1	0·65	0·68	0·6
10	0·67	0·65	0·66
100	6·3	3·3	4·8

UV^R - UV resistant

MTC^R - Mitomycin C resistant

* - Expected frequency of transformants if the two markers were unlinked

10 min). The amount of TCA-insoluble radioactivity was measured on filter paper discs. As a control the amount of DNA taken up by cells killed by 70°C for 15 min was also measured. Radioactivity was taken up by the wild type strain and strain 112 to a comparable extent which greatly exceeded the uptake by heat killed bacteria (Table 10) indicating that strain 112 was not defective in DNA uptake. DNase treatment reduced the background radioactivity because free DNA is retained on the filter discs and because in the first stages of transformation DNA attaches to the exterior of *D.radiodurans* but remains accessible to DNase (Tirgari, S. Ph.D. Thesis, Edinburgh 1978).

Determination of the number of inactivated DNA repair genes

It has previously been demonstrated that transformation of a mutant strain of *D.radiodurans* with donor DNA extracted from the same strain fails to restore the defective gene in the recipient. However, DNA extracted from other mutant strains is able to restore the defective gene. Several DNA repair genes have been identified in *D.radiodurans* in this way where DNA carrying one mutation will 'complement' the defect in another strain indicating that the two mutations are in different genes (Moseley and Copland 1978; Kitayama *et al.*, 1983). The same technique is used here to determine the number of defective genes represented in strains UVS9, UVS25, UVS78, 8, 112, 128 and 131. DNA was extracted from rifampicin resistant derivatives of these strains and used to transform each of the remaining strains to UV resistance. The UV dose used for selection and the time allowed for gene expression depended

Table 10

The uptake of ^{3}H DNA by the *D.radiodurans* wild type strain and strain 112.

Strain	Amount of radioactivity remaining on filter discs (cpm)	
	Before DNase treatment	After DNase treatment
Wild Type	8103	4364
Wild Type*	2925	38
112	7468	4009
112*	2146	44

* Indicates that the cells were heat killed

upon the UV sensitivity and the doubling time of the recipient strain. The selective dose was $250Jm^{-2}$ when strains UVS78, 128, 131, 261 and 301 were recipients and $450Jm^{-2}$ when strains 8, UVS9, 25 and 112 were recipients. Five hours was allowed for gene expression in strains 78, 261 and 301 and 10 hours for the remaining strains which all had doubling times of about 170 min, twice that of the former strains. UV-resistant transformants were restreaked and reirradiated with the original selective dose. The frequencies of transformants obtained are summarized in Table 11. Identification of UV-resistant transformants of strain 8 proved difficult and unreliable because of the relative resistance of this strain to UV. Strain 112 could not be used as a recipient because it was non-transformable.

In every instance the frequency of UV-resistant clones obtained by transformation of one strain with DNA from another exceeded the spontaneous mutation frequencies to UV resistance and the frequencies obtained by transformation of a strain with its own DNA, indicating that each mutation was in a different gene. The control frequency of transformation to rifampicin resistance varied in different transformations unpredictably and appeared to be partly a function of the source of the DNA but the reason for the variation was not investigated. The defective genes in the DNA repair-deficient strains were labelled as follows:-

Strain	Mutant Genes	Strain	Mutant Genes
UVS9	*mtcA* *uvsC*	128	*mtcA* *uvsF*
91	*uvsC*	1281	*uvsF*

Table 11

Frequencies of UV and rifampicin resistant transformants obtained from DNA repair-deficient strain of *D.radiodurans* using DNA from the same and other DNA repair-deficient strains.

* data from Moseley and Copland 1978 included for comparison.

Strains Donating DNA for Transformation		Recipient Strains										
		Wild Type	UVS9	UVS25	UVS78	112	131	128	8	301	261	Resistance to
	Wild Type	5.0×10^{-3}	7.7×10^{-5}	1.5×10^{-4}	1.6×10^{-4}	1.3×10^{-7}	1.5×10^{-4}	1.8×10^{-4}	2.0×10^{-3}	$^{*}4.6 \times 10^{-4}$	$^{*}1.7 \times 10^{-4}$	Rifampicin
		N/A	3.2×10^{-4}	4.0×10^{-5}	2.3×10^{-4}	1.0×10^{-7}	1.0×10^{-4}	1.1×10^{-3}	N/O	$^{*}5.8 \times 10^{-6}$	$^{*}3.4 \times 10^{-5}$	UV
	UVS9	2.5×10^{-3}	3.2×10^{-5}	7.9×10^{-6}	7.1×10^{-4}	ND	4.8×10^{-4}	2.3×10^{-4}	ND	2.7×10^{-5}	1.9×10^{-5}	Rifampicin
		N/A	$< 4.7 \times 10^{-6}$	7.5×10^{-5}	1.8×10^{-4}	ND	2.9×10^{-4}	5.0×10^{-4}	ND	4.4×10^{-5}	3.1×10^{-5}	UV
	UVS25	1.3×10^{-3}	1.7×10^{-4}	8.3×10^{-6}	1.7×10^{-4}	ND	5.0×10^{-4}	2.7×10^{-4}	ND	1.5×10^{-5}	2.2×10^{-5}	Rifampicin
		N/A	2.4×10^{-4}	$< 5.2 \times 10^{-6}$	3.6×10^{-4}	ND	3.2×10^{-5}	5.5×10^{-4}	ND	4.3×10^{-5}	4.3×10^{-5}	UV
	UVS78	2.5×10^{-3}	7.3×10^{-5}	2.3×10^{-5}	2.0×10^{-4}	ND	4.0×10^{-6}	7.1×10^{-6}	ND	5.0×10^{-3}	4.4×10^{-5}	Rifampicin
		N/A	3.9×10^{-5}	1.1×10^{-4}	$< 1.7 \times 10^{-7}$	ND	1.7×10^{-4}	8.8×10^{-5}	ND	5.0×10^{-4}	5.7×10^{-6}	UV
	112	5.0×10^{-3}	1.6×10^{-4}	2.0×10^{-4}	1.2×10^{-4}	ND	2.3×10^{-4}	3.1×10^{-4}	ND	1.1×10^{-5}	2.4×10^{-6}	Rifampicin
		N/A	2.8×10^{-5}	9.5×10^{-4}	6.0×10^{-5}	ND	2.7×10^{-4}	3.3×10^{-4}	ND	1.0×10^{-5}	1.1×10^{-5}	UV
	131	5.0×10^{-4}	1.8×10^{-4}	2.7×10^{-4}	2.2×10^{-5}	ND	5.0×10^{-4}	7.1×10^{-6}	2.0×10^{-5}	1.9×10^{-3}	2.1×10^{-5}	Rifampicin
		N/A	1.3×10^{-3}	1.9×10^{-4}	1.2×10^{-5}	ND	$< 1.0 \times 10^{-7}$	9.1×10^{-5}	N/O	5.0×10^{-4}	2.8×10^{-4}	UV
	128	2.0×10^{-3}	ND	ND	ND	ND	6.7×10^{-3}	2.4×10^{-4}	2.3×10^{-3}	6.0×10^{-5}	2.6×10^{-4}	Rifampicin
		N/A	ND	ND	ND	ND	3.4×10^{-4}	$< 1.0 \times 10^{-7}$	N/O	9.5×10^{-6}	2.3×10^{-4}	UV
	8	3.0×10^{-3}	1.1×10^{-4}	2.3×10^{-4}	1.8×10^{-4}	ND	1.3×10^{-3}	1.2×10^{-3}	3.0×10^{-4}	2.1×10^{-4}	2.1×10^{-4}	Rifampicin
		N/A	2.7×10^{-5}	1.0×10^{-4}	8.6×10^{-5}	ND	4.1×10^{-3}	1.8×10^{-4}	N/O	3.9×10^{-5}	4.2×10^{-4}	UV
	301	6.4×10^{-3}	4.5×10^{-4}	3.2×10^{-4}	6.8×10^{-5}	ND	3.8×10^{-4}	8.8×10^{-6}	1.8×10^{-4}	$^{*}7.1 \times 10^{-4}$	$^{*}3.0 \times 10^{-4}$	Rifampicin
		N/A	5.5×10^{-4}	2.9×10^{-4}	7.0×10^{-6}	ND	2.0×10^{-5}	8.5×10^{-5}	N/O	$<^{*}1.0 \times 10^{-8}$	$^{*}2.5 \times 10^{-5}$	UV
	261	3.0×10^{-4}	6.6×10^{-4}	5.9×10^{-4}	1.2×10^{-4}	ND	2.1×10^{-4}	1.2×10^{-5}	9.0×10^{-5}	$^{*}6.2 \times 10^{-4}$	$^{*}2.7 \times 10^{-4}$	Rifampicin
		N/A	2.8×10^{-4}	4.3×10^{-4}	1.7×10^{-3}	ND	6.3×10^{-5}	9.1×10^{-5}	N/O	$^{*}1.0 \times 10^{-6}$	$<^{*}1.0 \times 10^{-8}$	UV

N/A – Not Applicable ND – Not Determined N/O – None Obtained

Strain	Mutant Genes		Strain	Mutant Genes	
UVS25	*mtcA*	*uvsD*	131	*mtcA*	*uvsG*
251		*uvsD*	1311		*uvsG*
UVS78	*mtcA*	*uvsE*	8	*mtcA*	*uvsH*
781		*uvsE*	112	*mtcA*	*rec-1*

MMS-induced lethality and mutation in DNA repair-deficient strains

Measurement of the survival of the DNA repair-deficient strains after exposure to different MMS doses confirmed that those strains previously identified as 'MMS resistant' after exposure to a single MMS dose were as resistant as the wild type and therefore that repair of MMS-induced DNA-alkylation damage is unaffected by the mutations in these strains (Fig. 14).

The MMS-sensitive strains could conveniently be distinguished from MMS-resistant strains by the failure of the former to grow on TGY agar containing 0.02% MMS. The sensitivity of these strains varied presumably as a reflection of the different mutations but appeared, in the transformable strains, to be independent of the presence of the *mtcA* gene. The D_{37} doses for survival of strains 131 and 1311 were approximately 22-fold lower than that of the wild type strain and strain 131A, and similar to those of strains 8, 301 and 303 (Fig.15). Strains 112, 128 and 1281 were less sensitive with D_{37} doses approximately 11-fold lower than the wild type strain and strain 128A (Fig.15).

The *mtcA* gene is known to be involved in the removal of

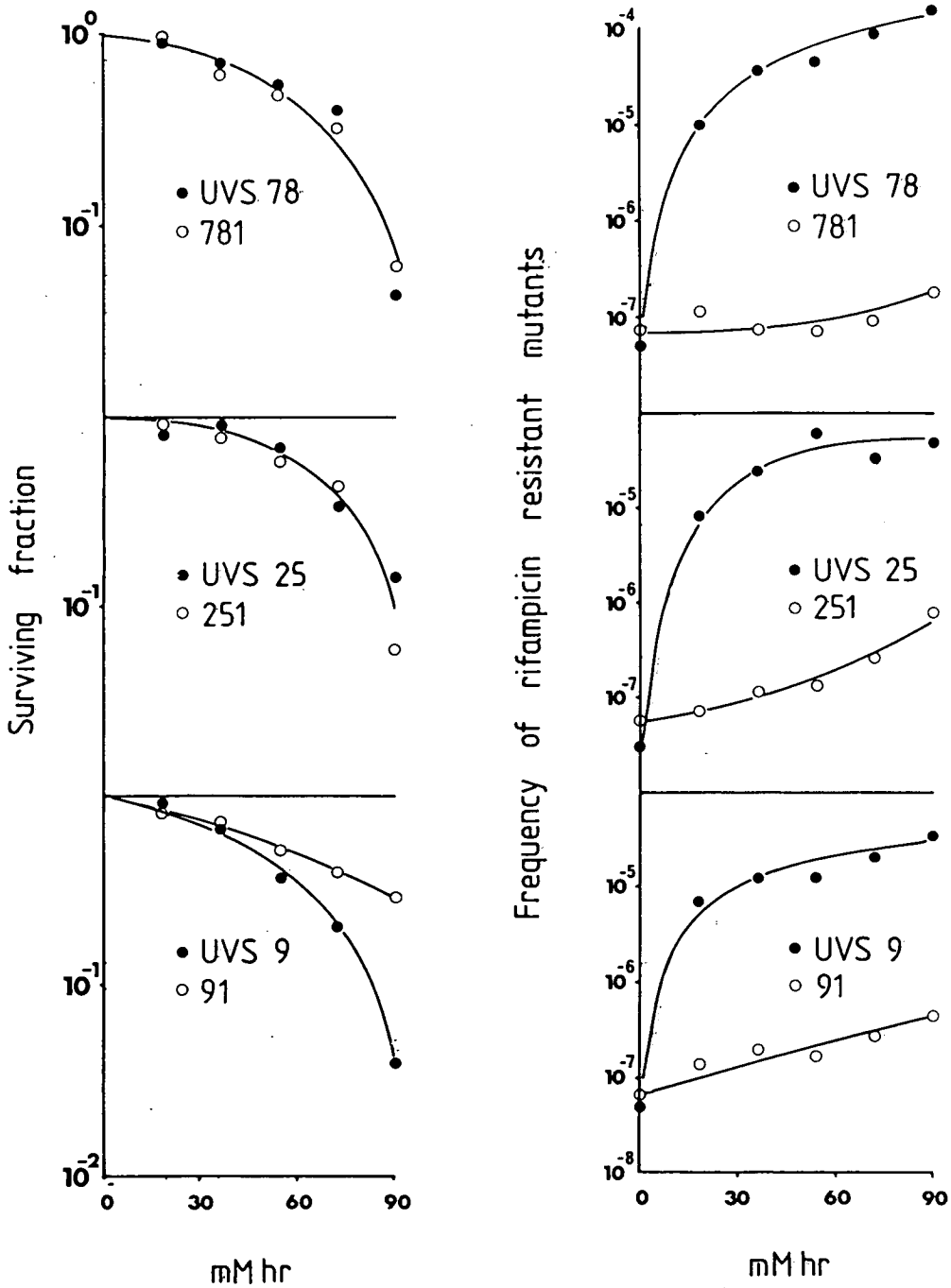


Figure 14

Survival and mutation of DNA repair-deficient *D.radiodurans* strains after exposure to methyl methanesulphonate.

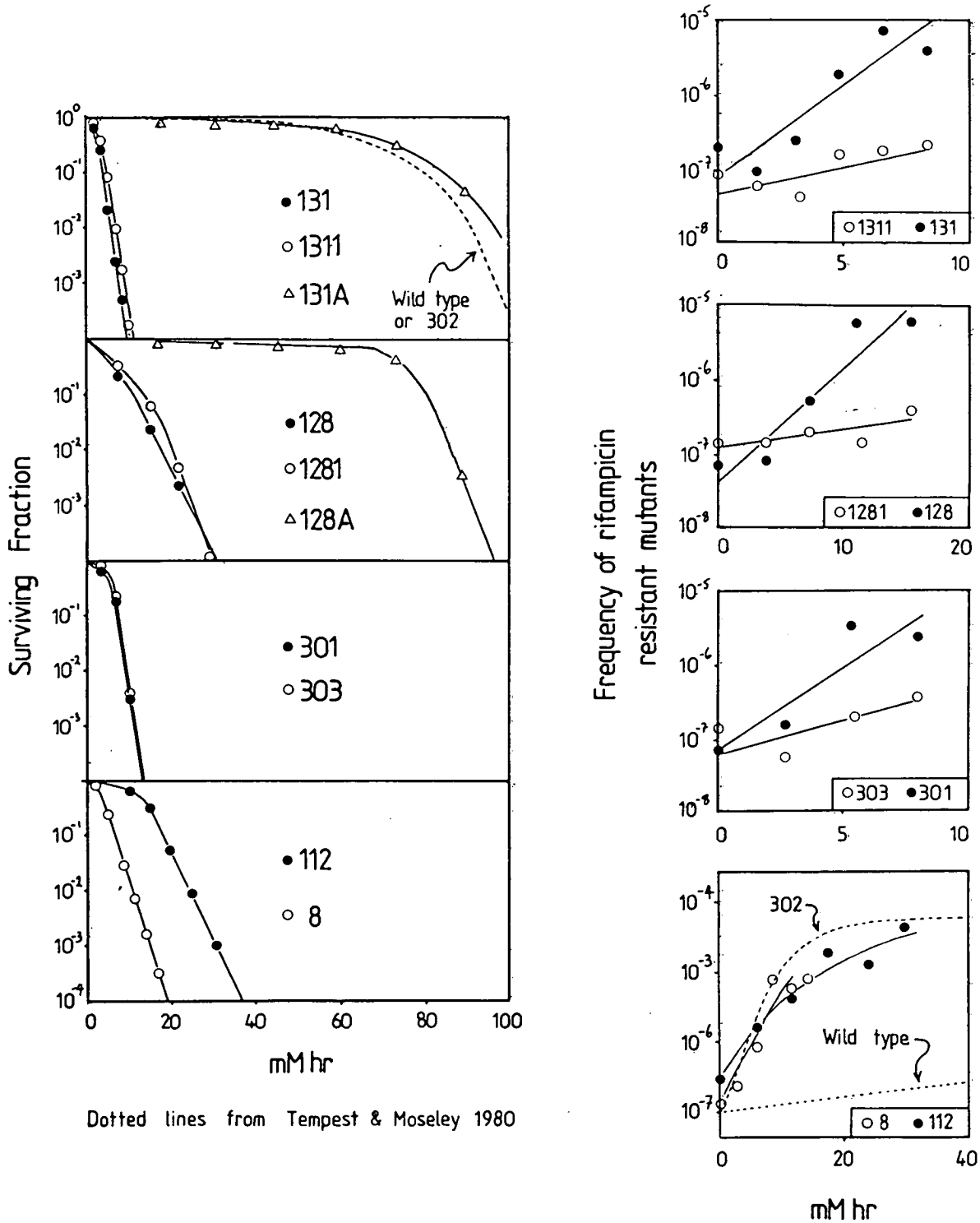


Figure 15

Survival and mutation of DNA repair-deficient *D.radiodurans* strains after exposure to methyl methanesulphonate.

mutagenic DNA alkylation damage in *D.radiodurans* so that strains lacking a functional *mtcA* gene are hypermutable by alkylating agents (Tempest and Moseley 1978). Measurement of the degree of mutation in the DNA repair-deficient strains after exposure to MMS revealed that the frequency of mutation to rifampicin resistance was only enhanced above the wild type level when the *mtcA* gene was mutant. The mutations in the remaining DNA repair genes are therefore not directly involved in the removal of mutagenic MMS-induced DNA alkylation damage.

UV-induced lethality and mutation in DNA repair-deficient strains

The survival of the DNA repair-deficient strains after UV irradiation varied according to which DNA repair gene was defective (Fig.12). Strains 91, 251 and 781 were relatively UV resistant in comparison with strains UVS9, UVS25 and UVS78, ie reintroduction of the functional *mtcA* gene into strains UVS9, UVS25 and UVS78 restored UV-resistance even though the mutation which created the UV-sensitive phenotype from the UV-resistant *mtcA*$^-$ background is still present. Similar observations were made by Moseley (Moseley and Evans 1983). Strains 91, 251 and 781 were also found to be able to excise TT and CT (TC) dimers, although at a slower rate than that achieved by the wild type strain (Fig.9). Two mutations are therefore required to eliminate excision repair of pyrimidine dimers in *D.radiodurans*; one must be in the *mtcA* gene and one in any one of the *uvsC*, *uvsD* or *uvsE* genes. Strains singly mutant in any of these are excision proficient and hence UV resistant.

The UV resistance of strains 91, 251 in which the *mtcA*

gene is wild type was not the same as the wild type and strain 302. Strains 91 (D_{37} 450Jm^{-2}) and 781 (D_{37} 300Jm^{-2}) were 1.3-fold and 1.9-fold more sensitive to UV than the wild-type strain whereas strain 251 (D_{37} 700Jm^{-2}) was 1.2-fold more resistant than the wild-type strain.

In contrast to the MMS-resistant strains, the UV sensitivity of the MMS-sensitive strains was produced by the inactivation of single genes and was independent of the *mtcA* gene. Strains 1281 and 1311 that possess a functional *mtcA* gene were approximately as UV sensitive as strains 128 and 131 respectively in which the *mtcA* gene is mutant. Strains 131 (D_{37} 14Jm^{-2}) and 1311 (D_{37} 14Jm^{-2}) were approximately 40-fold more sensitive to UV than the wild type. Strains 128 (D_{37} 28Jm^{-2}) and 1281 (D_{37} 46Jm^{-2}) were slightly more resistant than strains 131 and 1311 and were 12 and 20-fold more UV-sensitive than the wild type strain respectively. Strains 131 and 1311 differed from strains 128 and 1281 in lacking a shoulder to their survival curves. Strain 112 (D_{37} 130Jm^{-2}) has a shoulder to its survival curve and was the most UV resistant of the MMS-sensitive strains being 4-fold more UV sensitive than the wild-type strain. The shape of the survival curve of strain 8 (D_{37} 46Jm^{-2}) was unusual in being 'S' shaped which is indicative of a mixed culture. However, a single colony of strain 8 obtained from the first survival curve also had the same UV-survival curve.

The *D.radiodurans* wild-type strain and strains UVS9, UVS25 and UVS78 are non-mutable by UV (Tempest and Moseley 1982; Moseley and Evans 1983). The level of mutation produced after UV irradiation of the strains described in this section

was tested to determine the effect of the various mutations on UV mutability. The 10^2 dilutions from the UV survival curves (dilutions were in TGY broth) were grown for 18h then diluted and plated on TGY agar containing rifampicin ($20\mu gml^{-1}$). The frequency of rifampicin-resistant mutants which arose did not rise above the spontaneous frequency in any instance (data not shown). Thus none of the genes described above was responsible for maintaining the UV immutability of *D.radiodurans*.

Mitomycin C-induced lethality and mutation in DNA repair deficient strains

The *mtcA* gene is in part responsible for the repair of mitomycin C-induced crosslinks (Moseley and Copland 1978; Kitayama *et al.*, 1983). The mitomycin C sensitivity of strains UVS9, UVS25 and UVS78 was similar to the parent strain 302 suggesting that the additional mutations in these strains were not involved in the residual repair of mitocycin C crosslinks. However, restoration of the *mtcA* gene in strains 91, 251 and 781 increased the resistance to mitomycin C but did not completely restore it to wild type levels (Fig.13), suggesting that the *uvsC*, *uvsD* and *uvsE* genes were also partly responsible for mitomycin C crosslink repair. Strain 781 (D_{37} $90\mu gml^{-1}$ min), the most sensitive of the three strains (also the most UV sensitive), was 2.4-fold more sensitive than the wild type strain, whereas strains 91 (D_{37} $150\mu gml^{-1}$ min) and 251 (D_{37} $110\mu gml^{-1}$ min) were 1.5- and 2.0-fold more sensitive respectively.

The mitomycin C-sensitivity of the MMS-sensitive strains differed from the MMS-resistant strains in that the sensitivity induced by mutation of the single *mtcA* (strain 302), *uvsF*

(strain 1281) and *uvsG* (strain 1311) genes was additive, i.e. mutations in both the *mtcA* and *uvsF* genes (strain 128) or the *mtcA* and *uvsG* genes (strain 131) produced a phenotype which was more mitomycin C sensitive than that produced by either single mutant gene. Strains 1281 (D_{37} 23μgml^{-1} min) and 1311 (D_{37} 52μgml^{-1} min) were 9 and 4-fold more sensitive than the wild type whereas strains 128 (D_{37} 4μgml^{-1} min) and 131 (D_{37} 4μgml^{-1} min) were 55-fold more sensitive than the wild type strain. Some residual capacity for repair of mitomycin C crosslinks remains in the latter strains however since strain 8 (D_{37} 0.7μgml^{-1} min) is 314 fold more sensitive than the wild type strain.

The induction of mutation to rifampicin resistance by mitomycin C was measured as for UV; in no strain did the number of rifampicin-resistant mutants rise above spontaneous mutation levels (data not shown).

A summary of the origins and general phenotypes of the DNA repair deficient strains described above is given in figure 16.

Part B Incision of DNA in response to DNA damage

Incision of DNA in response to UV irradiation in MMS resistant strains

Typical profiles of the distribution of radioactivity-labelled DNA in alkaline sucrose gradients after UV irradiation of related representatives of the MMS-resistant strains of *D.radiodurans* are shown in Fig.17. The profiles for strains 91 and 251 were similar to 781 and those of strains UVS9 and

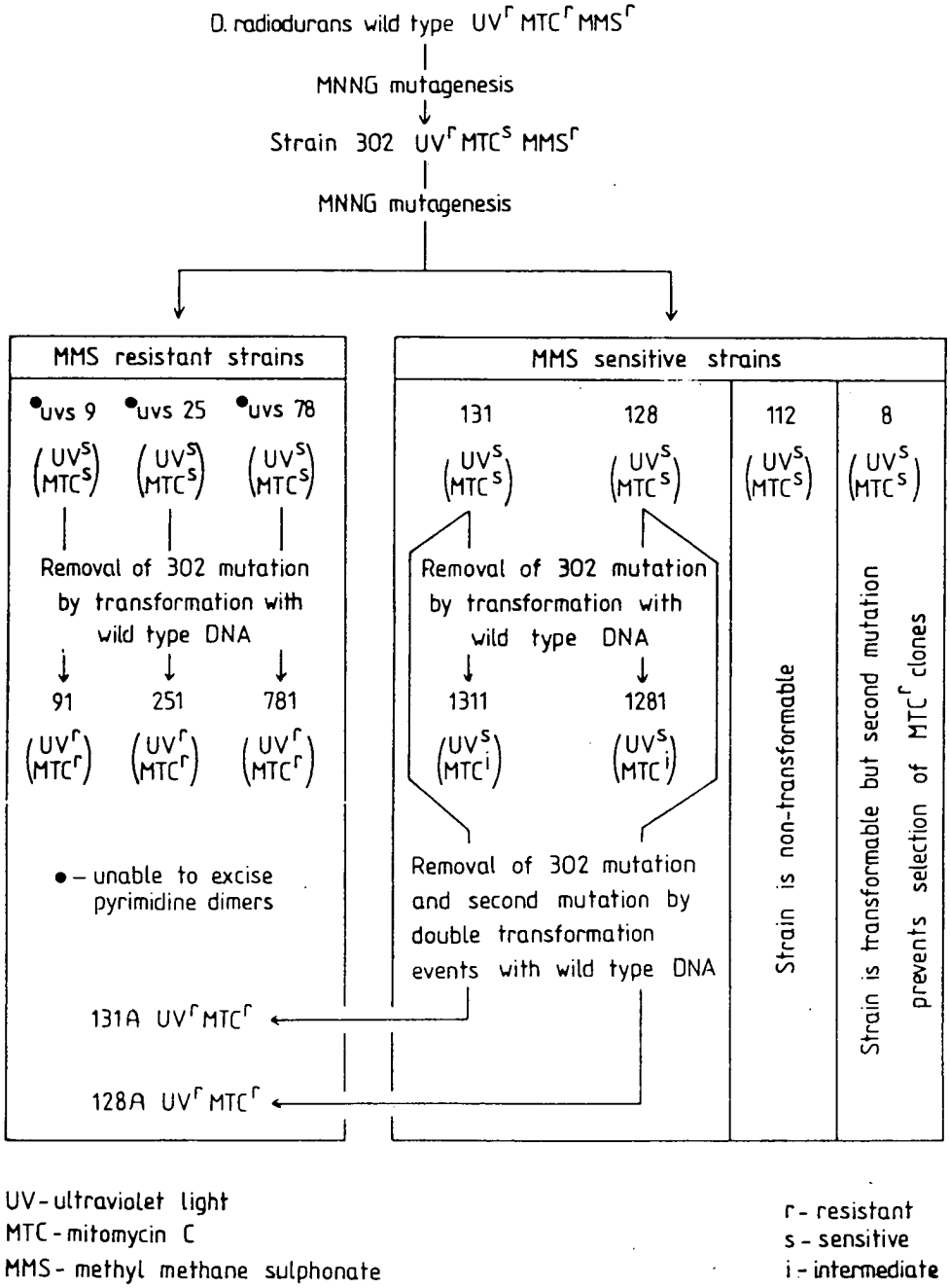


Figure 16

Summary of the origins of DNA repair-deficient *D. radiodurans* strains.

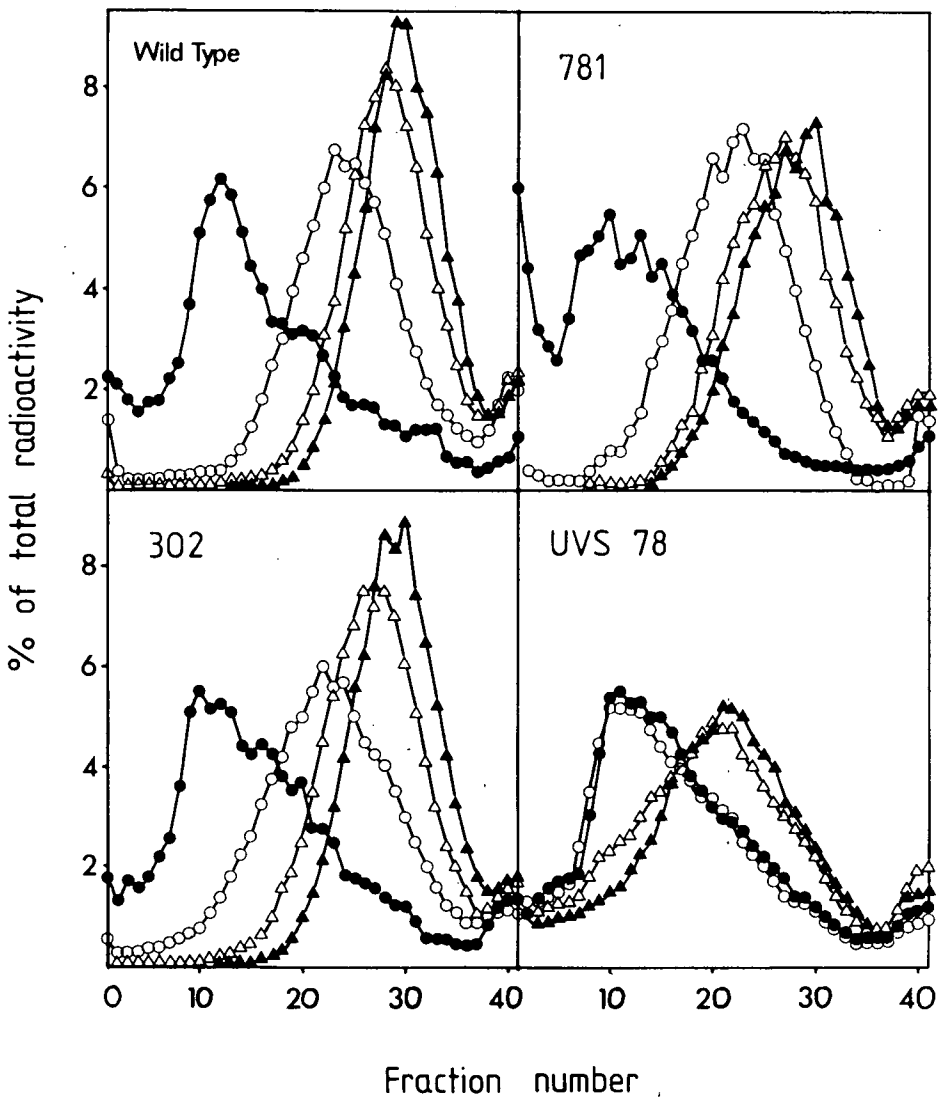
Figure 17

Typical sedimentation profiles in alkaline 5-20% sucrose gradients of DNA from the *D.radiodurans* wild type strain and representative MMS-resistant, DNA-repair-deficient strains.

Strain	Mutant Genotype
Wild type	-
302	*mtcA*
UVS78	*mtcA* *uvsE*
781	*uvsE*

(Strains 25 and 9 produce profiles similar to those of strain 78. Strains 251 and 9 produce profiles similar to those of strain 781).

● no UV o 125 Jm^{-2} Δ 250 Jm^{-2} ▲ 375 Jm^{-2}


Wild Type
781
302
UVS 78
% of total radioactivity
Fraction number
0
10
20
30
40
2
4
6
8

UVS25 were similar to those of UVS78. The production of DNA single strand breaks (DNA incisions) was only impaired in strains UVS9, UVS25 and UVS78 and was most clearly demonstrated at UV doses in the region of $125Jm^{-2}$ (Fig.18). At this dose strains UVS9, UVS25 and UVS78 all failed to produce single-strand breaks in their DNA, whereas the related strains where the *mtcA* gene was wild type all introduced as many single-strand breaks as the wild type strain (Figs.18 and 19). To check whether the failure to incise DNA in strains UVS9, UVS25 and UVS78 was absolute, strains were incubated for 2h after UV irradiation in TGY medium to allow a potentially partially-disabled incision enzyme to act. However no breaks were detected (Fig.18). The number of single-strand breaks, as well as the number of pyrimidine dimers introduced into DNA were linearly related to the UV dose so that the ratio of breaks to the initial number of dimers removed was approximately constant (Fig.19), e.g. 2.2% of the number of dimers after $125Jm^{-2}$ UV, 2.0% after $250Jm^{-2}$ and 1.4% after $375Jm^{-2}$ UV. This percentage would be lowered if $\hat{C}C$ dimers, which are not detected here, and which probably constitute a large fraction of the UV photoproducts in the DNA due to the high G+C content of *D.radiodurans* DNA (Patrick and Rahn 1975), are also included. Single-strand breaks were introduced into the DNA of strains UVS9, UVS25 and UVS78 at high UV doses but they were tenfold fewer per Jm^{-2} than in the wild type strain (Fig.19).

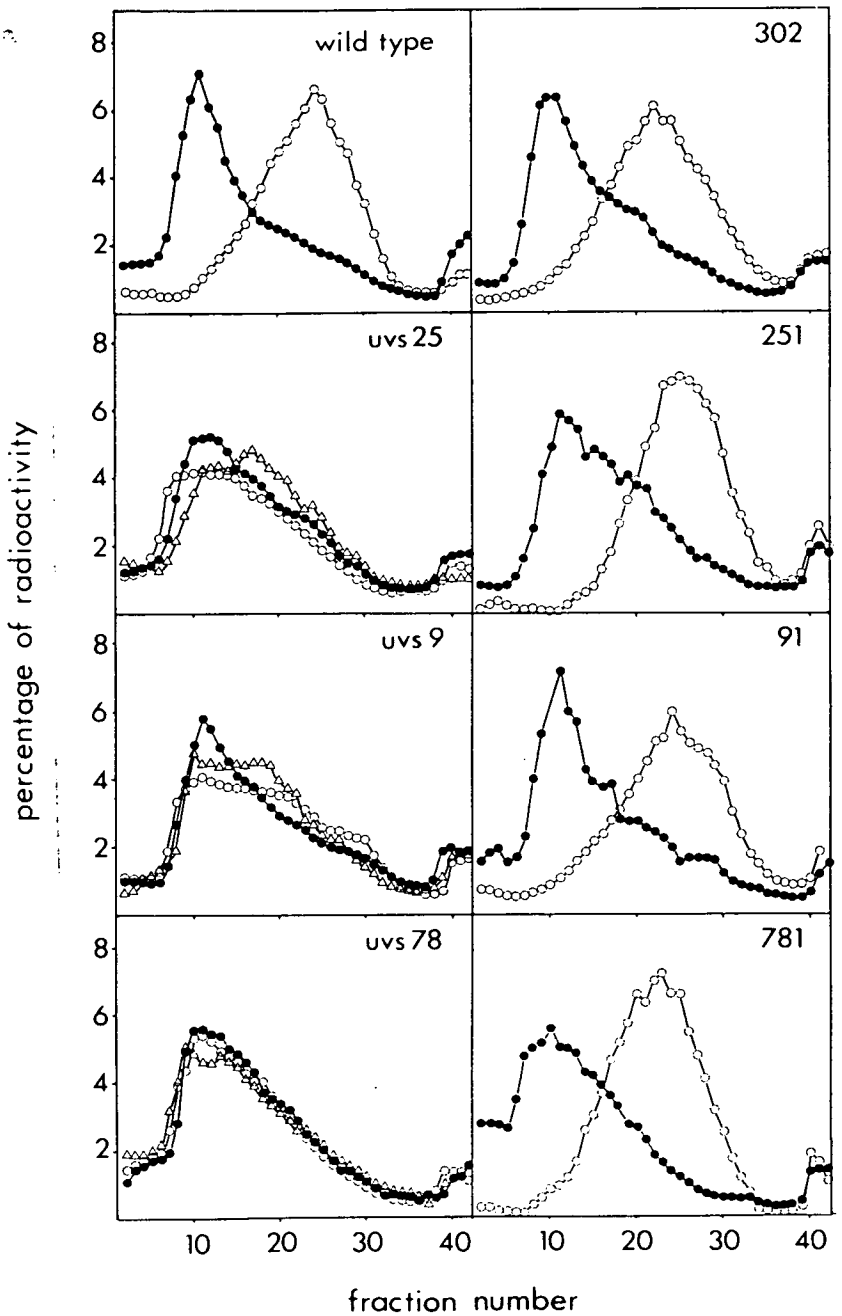
Incision of DNA in response to other DNA damage in MMS resistant DNA repair-deficient strains

These experiments were (a) to determine if the incision

Figure 18

Sedimentation profiles in alkaline 5-20% sucrose gradients of DNA from MMS-resistant DNA repair-deficient *D.radiodurans* strains after UV irradiation (125 Jm^{-2}).

- ● no UV
- o 125 Jm^{-1} UV
- Δ 125 Jm^{-2} UV plus 2 hours incubation in growth medium.


wild type
302
uvs 25
251
uvs 9
91
uvs 78
781
percentage of radioactivity
fraction number
2
4
6
8
10
20
30
40

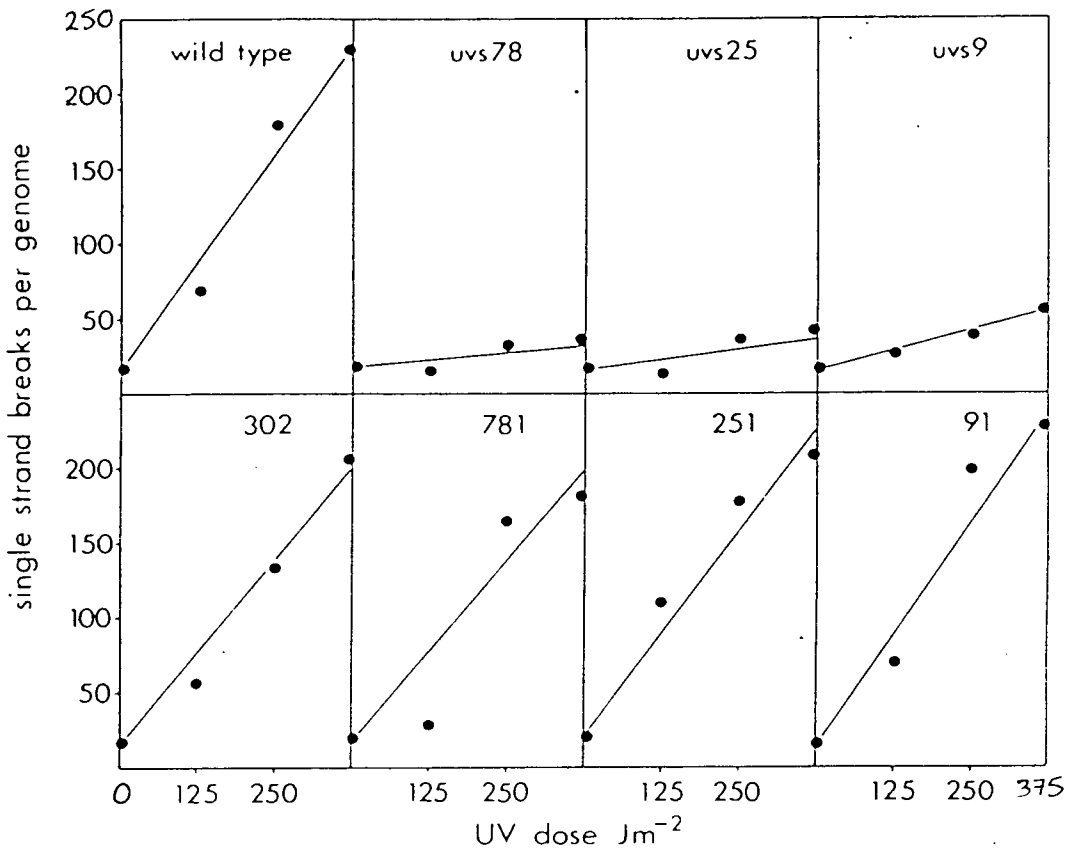


Figure 19

Number of single-strand DNA breaks produced per genome after UV irradiation of the wild type and MMS-resistant DNA repair-deficient strains.

The *D.radiodurans* genome size is taken as 2.0×10^9 daltons (Hansen 1978).

deficiency observed in some strains extended to a failure to incise DNA damaged with other lesions ie whether there were further incision enzymes in *D.radiodurans*, (b) to determine whether the substrate range of the UV incision systems could be extended, since the *mtcA* gene is known to be required for incision of DNA in response to bromomethylbenzanthracene adducts besides pyrimidine dimers (Tempest and Moseley 1980).

The error introduced by spontaneous strand-breakage at AP sites was assumed to be small enough to distinguish between an incision proficiency or deficiency since less than 2% of AP sites are incised under these gradient conditions (Lindahl and Anderson 1972). Spontaneous loss of alkylated bases which is the source of these AP sites is also negligible over the time course of the experiments (Margison and O'Connor 1973).

The UV incision-deficient strains UVS9, UVS25 and UVS78 produced comparable distributions of radioactivity in alkaline sucrose gradients to those produced by the wild type and strain 302 after exposure to MNNG, ENNG, MMS, EMS and NQO (Fig.20). The distributions varied slightly in shape between strains which would account for the differences in the calculated number of single-strand breaks in different strains after exposure to the DNA damaging agents (Table 12). The variation in the number of DNA incisions was not consistent with an incision defect in these strains comparable to that observed after UV irradiation. However, consistently fewer incisions were produced in response to hydroxylamine-induced damage in strains UVS9, UVS25 and UVS78 suggesting that a component of this damage was also substrate for the incision system absent

Figure 20

Sedimentation profiles in 5-20% alkaline sucrose gradients of DNA from wild type *D.radiodurans* and MMS-resistant repair deficient strains after exposure to various DNA damaging agents.

DNA damaging Agent	Dose
ENNG	750 $\mu g ml^{-1} min$
MNNG	750 $\mu g ml^{-1} min$
MMS	20 mM hr
EMS	100 mM hr
NQO	375 $\mu g ml^{-1} min$
HA	990 $mg ml^{-1} min$
DCMTC	330 $\mu g ml^{-1} min$

The arrows mark the peak position of undamaged DNA.

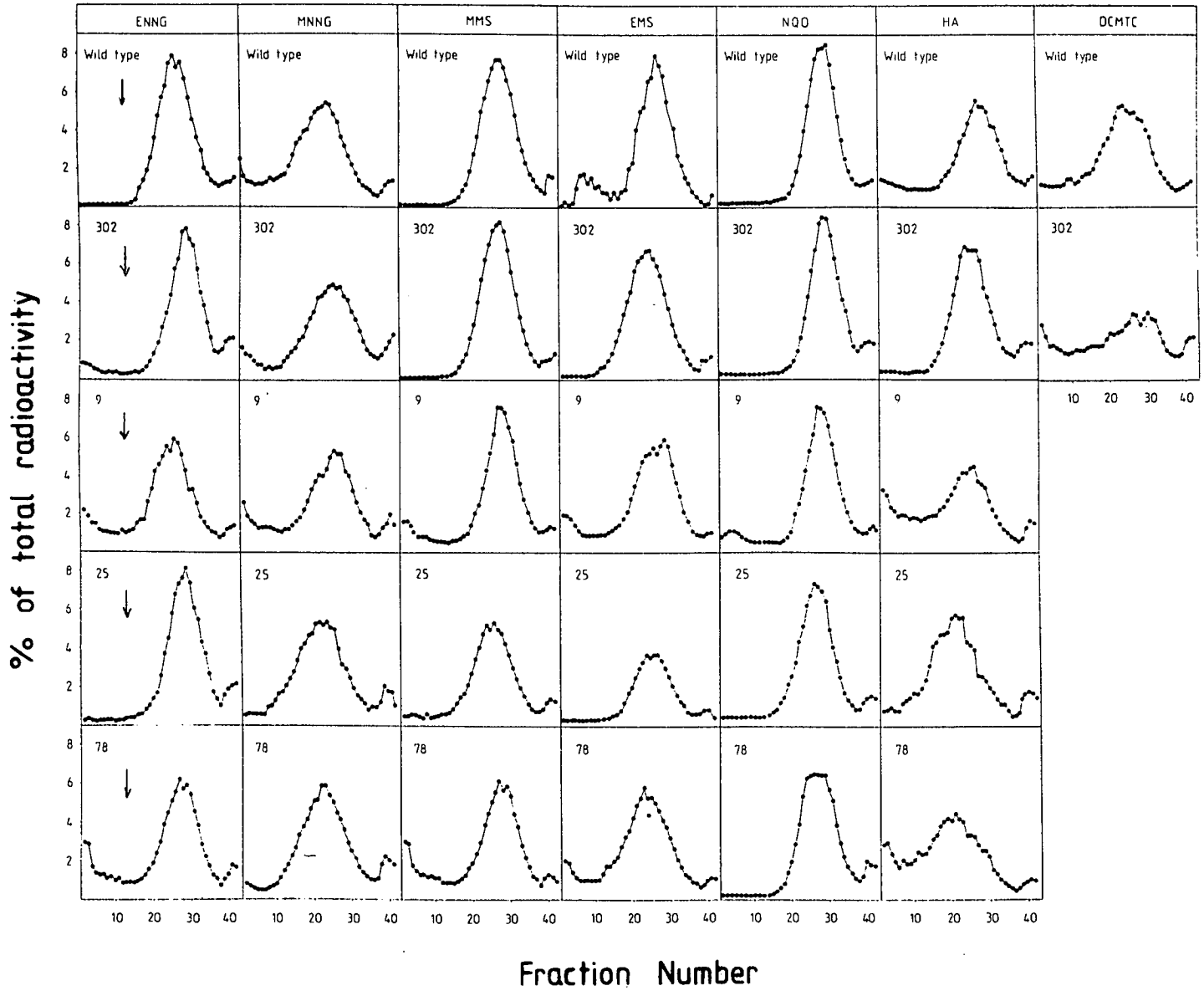

ENNG
MNNG
MMS
EMS
NQO
HA
DCMTC
Wild type
302
9
25
78
% of total radioactivity
Fraction Number

Table 12

The number of single strand breaks per genome calculated from the distribution of radioactivities shown in figure 20.

	Strain				
DNA damaging agent	Wild Type	302	UVS9	UVS25	UVS78
ENNG	133 (100%)	211 (158%)	200 (150%)	180 (135%)	200 (150%)
MNNG	83 (100%)	80 (96%)	118 (140%)	95 (114%)	100 (120%)
MMS	167 (100%)	133 (80%)	167 (100%)	117 (76%)	140 (84%)
EMS	125 (100%)	87 (70%)	125 (100%)	125 (100%)	95 (76%)
NQO	182 (100%)	217 (119%)	200 (110%)	125 (69%)	140 (77%)
HA	183 (100%)	140 (77%)	100 (55%)	71 (39%)	69 (38%)
DCMTC	111 (100%)	130 (117%)	ND	ND	ND

Abreviations: ENNG Ethylnitrosoguanidine
MNNG Methylnitrosoguanidine
MMS Methylmethanesulphonate
EMS Ethylmethanesulphonate
NQO Nitroquinoline oxide
HA Hydroxylamine
DCMTC Decarbomoyl mitomycin C

from these strains. Strain 302 also produced fewer breaks after exposure to hydroxylamine but the effect was not as great as in the other strains and did not conclusively implicate the *mtcA* gene in the incision of hydroxylamine-induced DNA damage. It is possible that the lack of a functional *mtcA* gene in conjunction with the *uvsC*, *uvsD* or *uvsE* genes may inhibit incision of a component of hydroxylamine-induced DNA damage in a manner similar to the inhibition produced in response to UV. Strain 302 incised DNA damaged with DCMTC which is surprising in view of the extreme sensitivity of the strain to DCMTC and the fact that the 302 mutation causes a failure to excise mitomycin C crosslinks from DNA (Kitayama *et al.*, 1983).

Similar measurements of the number of single-strand breaks produced in response to N-acetoxy-acetylaminofluorine (AAAF) by the method described by Tempest and Moseley (1980) in which AAAF was dissolved in DMSO revealed that the DMSO alone induced single-strand breaks in DNA. This occurred in the wild type and strain 78 to equal degrees suggesting that it was not caused by the UV-incision system. It also occurred in these strains in the presence of 20mM EDTA which is known to inhibit an endonuclease induced in *D.radiodurans* by certain alcohols such as butanol (Driedger and Grayston 1970) (Fig.21).

The effect of EDTA and protein synthesis on UV-induced DNA incisions

The UV-incision-proficient wild-type strain and strains 302, 91, 251 and 781 were allowed to stand at 4°C for 20 min

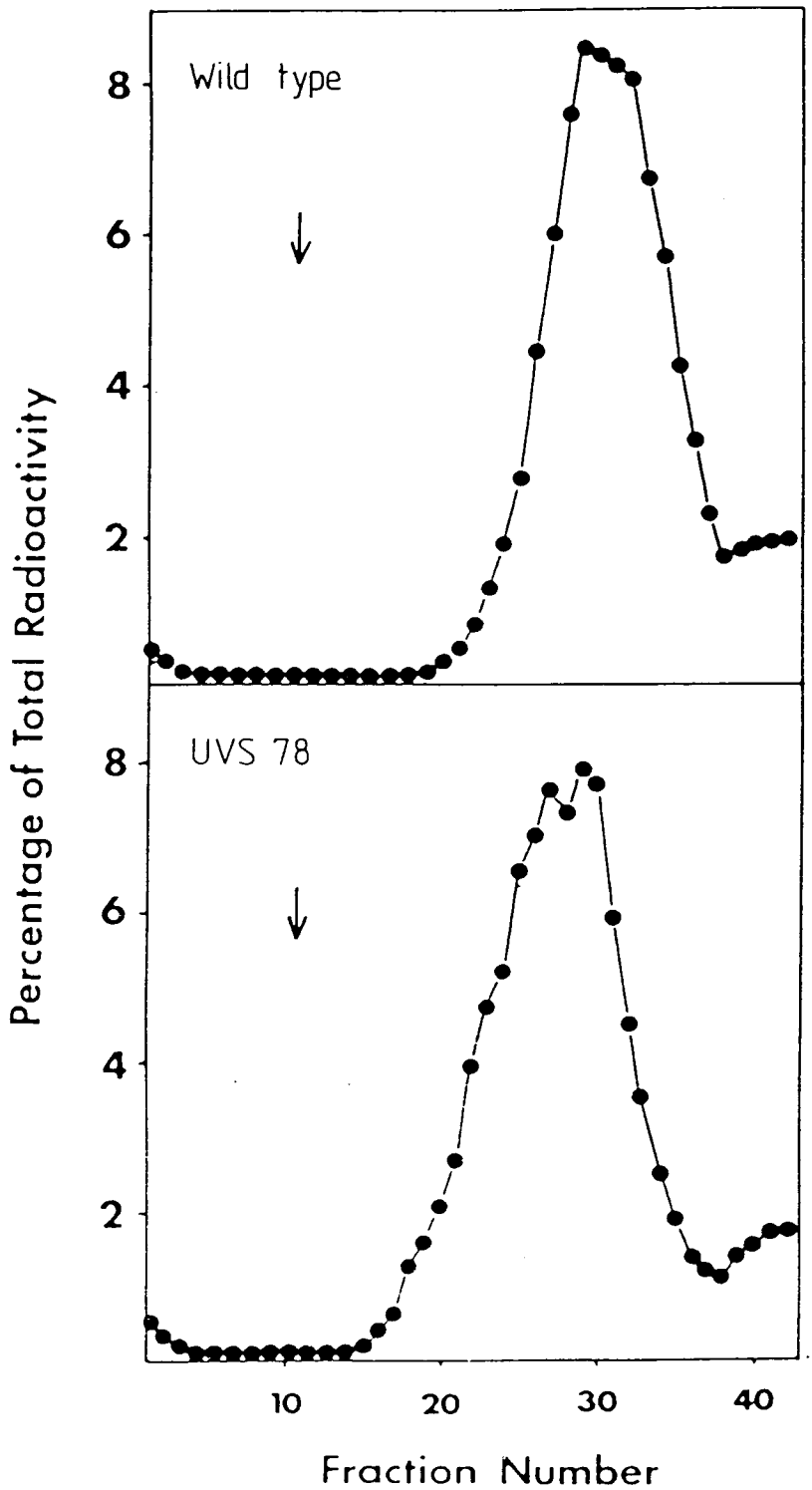


Figure 21

Sedimentation profiles in alkaline 5-20% sucrose gradients of DNA from the *D.radiodurans* wild type strain and strain UVS78 after exposure to dimethyl-sulphoxide (25%) in presence of EDTA (20mM).

The arrows mark the peak positions of untreated DNA.

before irradiation with 125Jm^{-2} in buffer containing EDTA (20 mM) or chloramphenicol (15μgml^{-1}) to allow complete absorption of cations or termination of protein synthesis. Neither EDTA nor the absence of protein synthesis prevented incision in response to UV in any strain (Fig.22).

DNA incision in response to UV in MMS-sensitive strains

Preliminary experiments established that strains 112, 128 and 131 produced DNA sedimentation profiles where the majority of the DNA was in the top few fractions of the alkaline sucrose gradients after UV irradiation. The MMS-sensitive strains 112, 128, 1281, 131 and 1311 were subsequently irradiated at very low doses (Fig.23). In all cases the number of single-strand breaks introduced was greater than that introduced by the MMS-resistant strains after a tenfold higher UV dose. The unirradiated control DNA in these strains had a broader distribution of molecular weight than unirradiated wild type control DNA. Strain 8 differed from the other MMS-sensitive strains in producing only as many single-strand breaks per Jm^{-2} of UV as the wild type strain.

Part C. DNA degradation in response to DNA damage

A consequence of the loss of excision repair in *uvrA*$^-$ and *uvrB*$^-$ *E.coli* strains is a large reduction in the amount of DNA degraded after UV irradiation (Boyce and Howard-Flanders 1964; Setlow 1967; Fong and Bockrath 1977). Other *E.coli* strains mutant in other genes, including *recA* (Setlow 1968), *polA* (Boyle *et al.*, 1970) or *uvrD* (Ogawa *et al.*, 1968),

Figure 22

Sedimentation profiles in alkaline 5-20% sucrose gradients of DNA from MMS-resistant DNA repair-deficient *D.radiodurans* strains after UV irradiation in the presence of chloramphenicol (15 $\mu g ml^{-1}$) or EDTA (20 mM).

- o no UV plus EDTA
- ∇ 125 Jm^{-2} UV plus EDTA
- ▲ no UV plus chloramphenicol
- ● 125 Jm^{-2} plus chloramphenicol

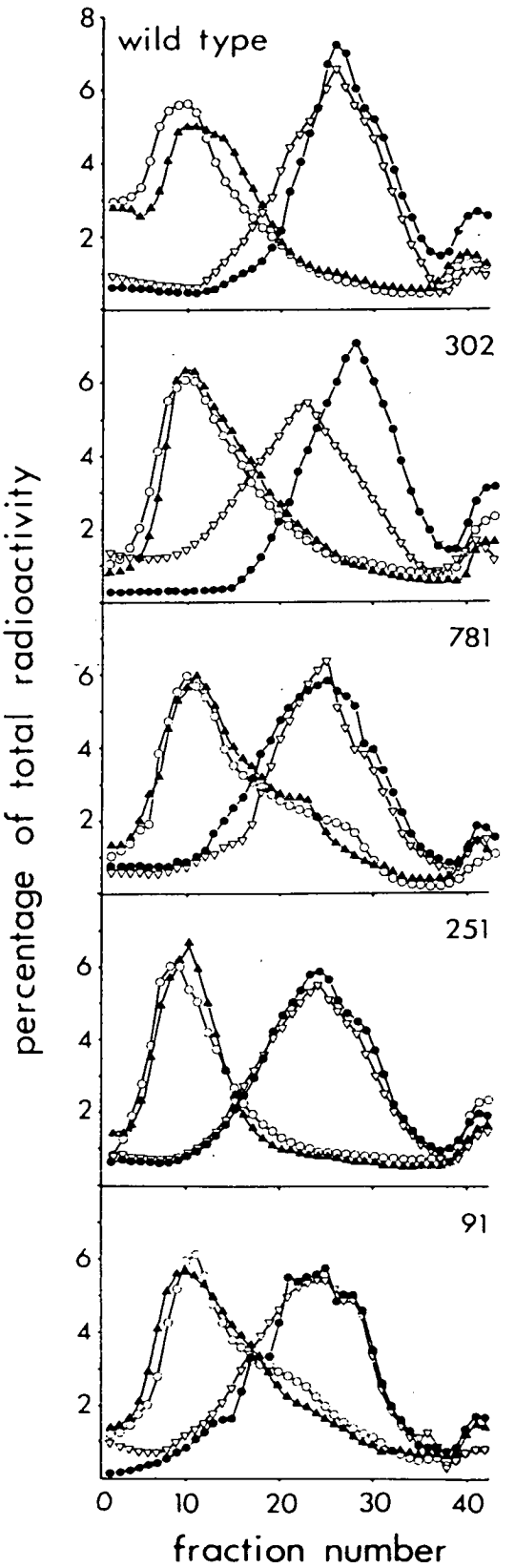
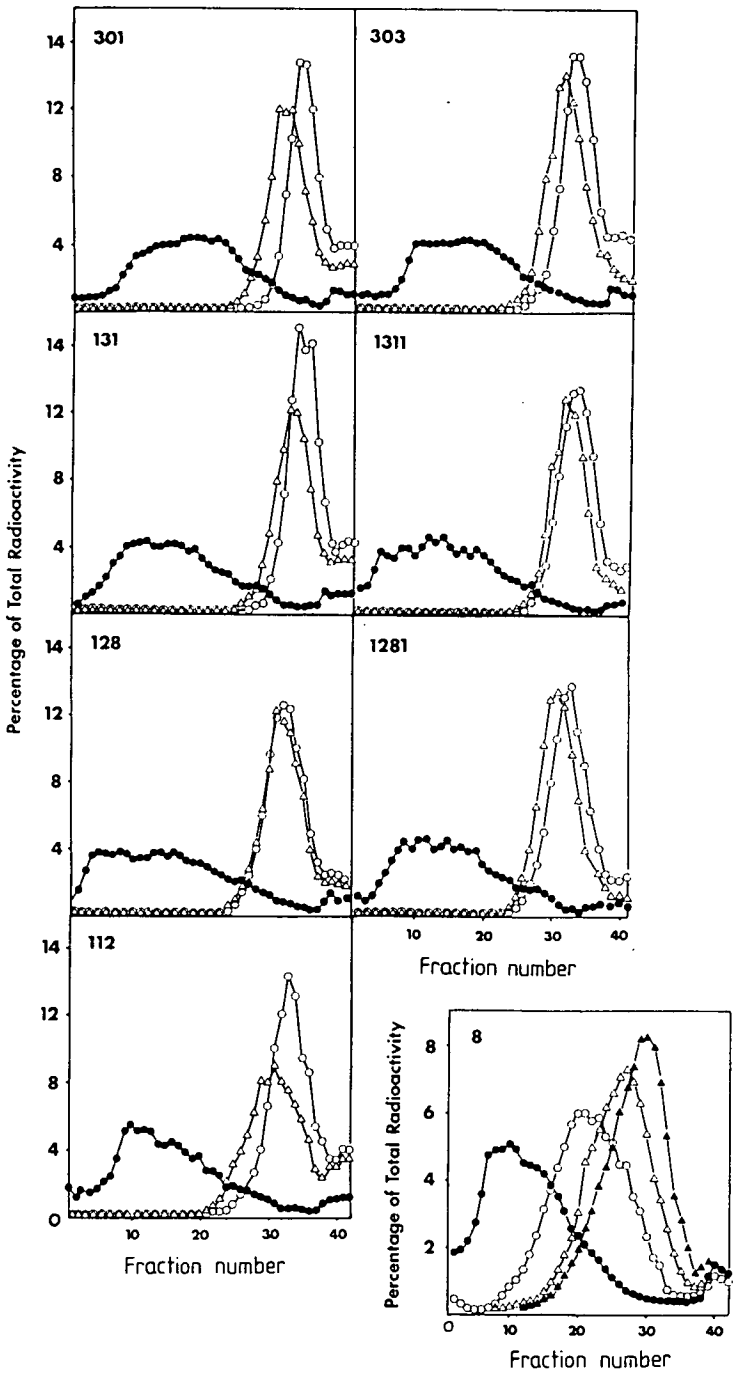

wild type
302
781
251
91
8
6
4
2
0
10
20
30
40
percentage of total radioactivity
fraction number

Figure 23

Sedimentation profiles in alkaline 5-20% sucrose gradients of DNA from MMS-sensitive *D.radiodurans* strains after UV irradiation.

● no UV Δ 15 Jm^{-2} UV o 30 Jm^{-2} UV	Strains 112, 128, 1281, 131, 1311, 301 & 303
● no UV o 125 Jm^{-2} UV Δ 250 Jm^{-2} UV ▲ 500 Jm^{-2} UV	Strain 8


301
303
131
1311
128
1281
112
8
Percentage of Total Radioactivity
Fraction number
Percentage of Total Radioactivity
Fraction number

"recklessly" degrade an excess of DNA after UV irradiation. *D.radiodurans* is known to degrade DNA in response to UV irradiation (Moseley 1967; Varghese and Day 1970) but this has not been quantified or correlated with DNA repair defects as in *E.coli*. The characteristics of the DNA degradation produced in the *D.radiodurans* wild type and DNA repair-deficient strains was therefore examined.

DNA degradation in MMS-resistant strains after UV irradiation

The quantity of ^{3}H-labelled DNA remaining in the TCA-insoluble cell fraction and the amount of ^{3}H-labelled degradation products released into the growth medium after UV irradiation of the *D.radiodurans* wild type strain is shown in Fig.24. The spread of data for intracellular DNA content is greater than that for release of the DNA degradation products and the *data* shown are therefore the average of five experiments. The amount of label lost from the TCA-insoluble fraction corresponds with the amount of label released into the medium, which agrees with previous data obtained after x-irradiation of *D.radiodurans* (Dean *et al.*, 1970; Fox and Hopkins 1970). The rate of DNA degradation is dose-independent at approx.5% of the total DNA per h while the amount of DNA degraded is dose-dependent at approx.1.2% of total DNA per $100Jm^{-2}$ UV. This is significantly less degradation per Jm^{-2} than in *E.coli* eg. 60 fold less after $50Jm^{-2}$ (Boyle *et al.*, 1970; Setlow 1968).

The characteristics of DNA degradation in DNA repair deficient *D.radiodurans* were different from the wild type strain. Strains UVS9, UVS25 and UVS78 degraded very little DNA relative to the wild type demonstrating that the majority of post-UV DNA

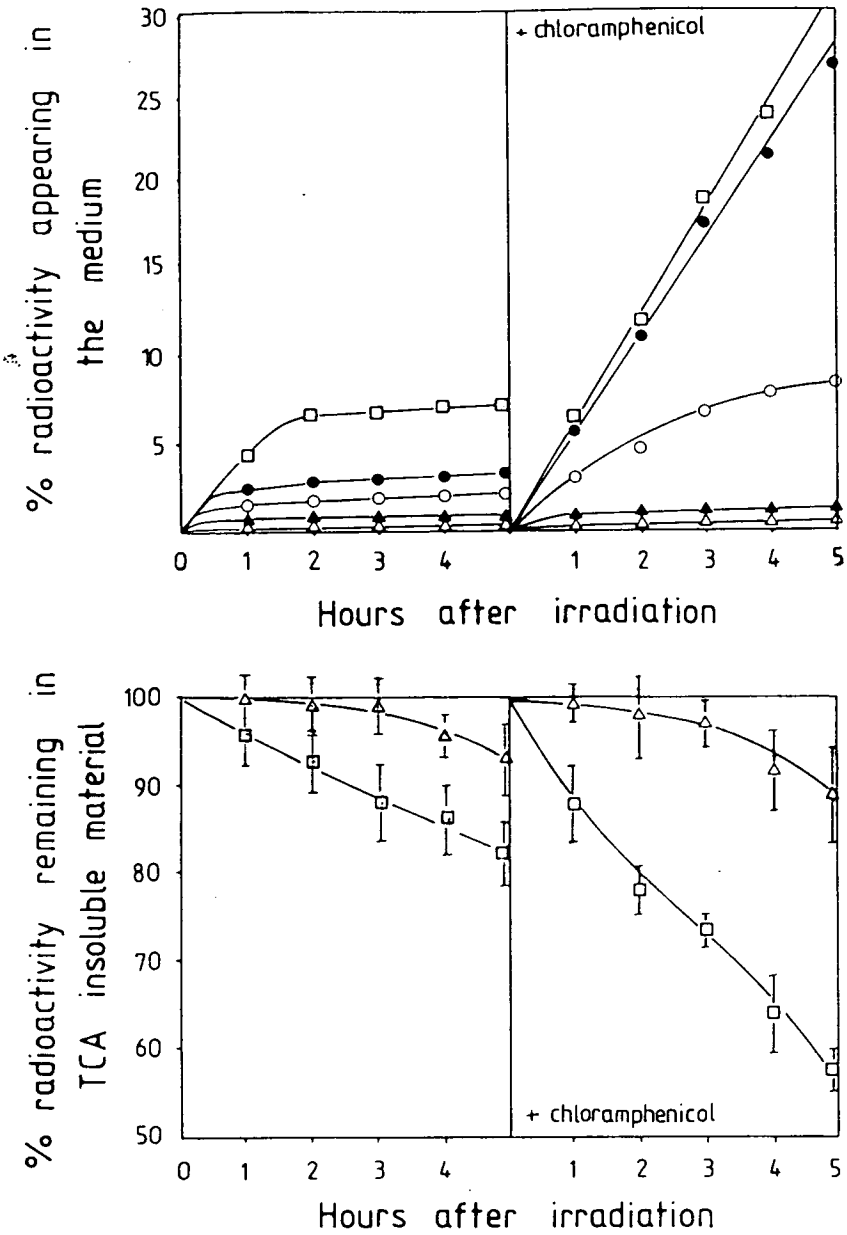


Figure 24

Amount of ^{3}H labelled DNA present as TCA insoluble material and in the growth medium in the presence or absence of chloramphenicol (15 $\mu g ml^{-1}$) after UV irradiation of the *D.radiodurans* wild type strain.

Δ no UV

▲ 60 Jm^{-2} UV

o 125 Jm^{-2} UV

● 250 Jm^{-2} UV

□ 500 Jm^{-2} UV

degradation in the wild type strain is due to excision repair mechanisms that are absent from these strains (Fig.25). The residual rate was dose-independent and proceeded at 0.04% of total DNA per h. The initial rate of degradation in strain 302 was slower than in the wild type and was dose-dependent. The amount of DNA degraded was similar to that degraded by the wild type below $300Jm^{-2}$ but was greater than in the wild type above this dose. Strains 91, 251 and 781 also displayed a dose-dependent rate of degradation unlike the wild type such that degradation continued for several hours after irradiation. However, the amount of DNA degraded was sometimes greater than that degraded by the wild type for the same UV dose.

DNA degradation in the MMS-sensitive strains after UV irradiation

The MMS-sensitive strains 112, 128, 1281, 131, 1311, 301 and 303 all spontaneously degraded DNA to a greater rate and extent than the wild type. In strains 131 and 1311 this was 0.8% h^{-1} and in the remaining strains 0.4% h^{-1}. Strain 8 behaved as the wild type strain both in the absence of UV irradiation and after UV irradiation. UV irradiation of the remaining strains (112, 128, 1281, 131, 1311, 301 and 303) at UV doses below $100Jm^{-2}$ produced enhanced dose-dependent rates and extents of DNA degradation in comparison with the wild type (Fig.26). The rates of degradation were proportional to the UV dose and were greatest in strains 131 and 112. Above $100Jm^{-2}$ the rate in all these strains was dose-independent at 8% h^{-1}. The final extent of the DNA degradation was not determined in most instances but in strains 301 and 303, above doses of $120Jm^{-2}$,

Figure 25

Release of radioactivity from ^{3}H labelled DNA into the growth medium by the MMS-resistant, DNA repair-deficient *D.radiodurans* strains after UV irradiation.

o no UV

● 125 Jm^{-2} UV

Δ 250 Jm^{-2} UV

▲ 500 Jm^{-2} UV

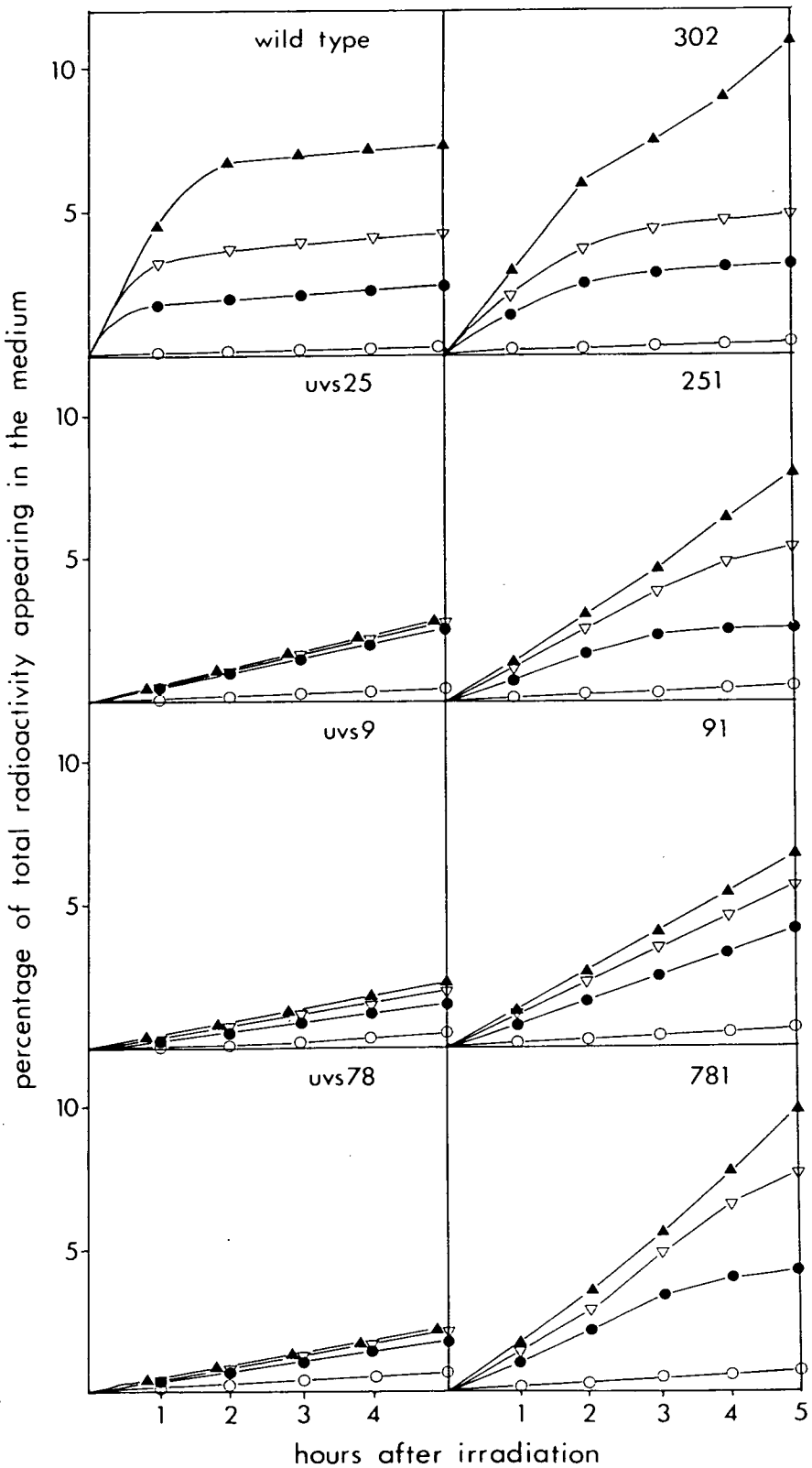
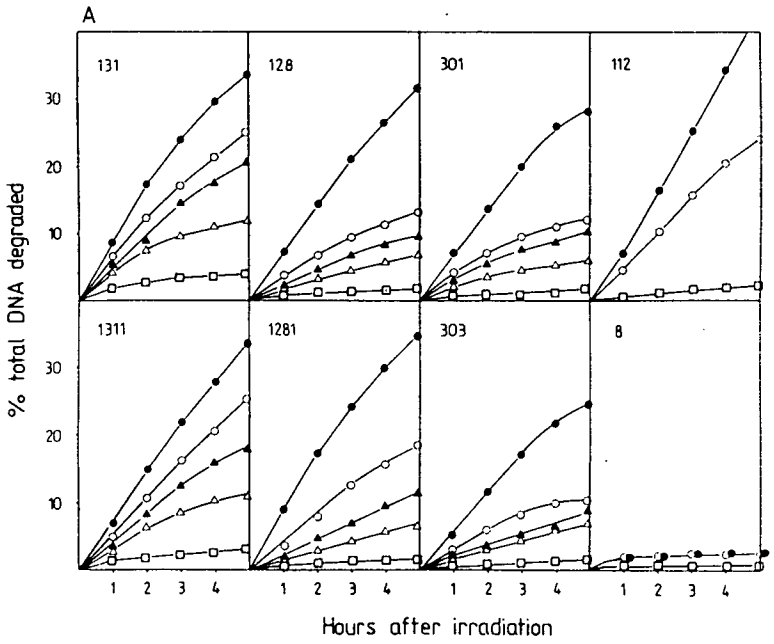

wild type
302
uvs25
251
uvs9
91
uvs78
781
10
5
1
2
3
4
5
percentage of total radioactivity appearing in the medium
hours after irradiation

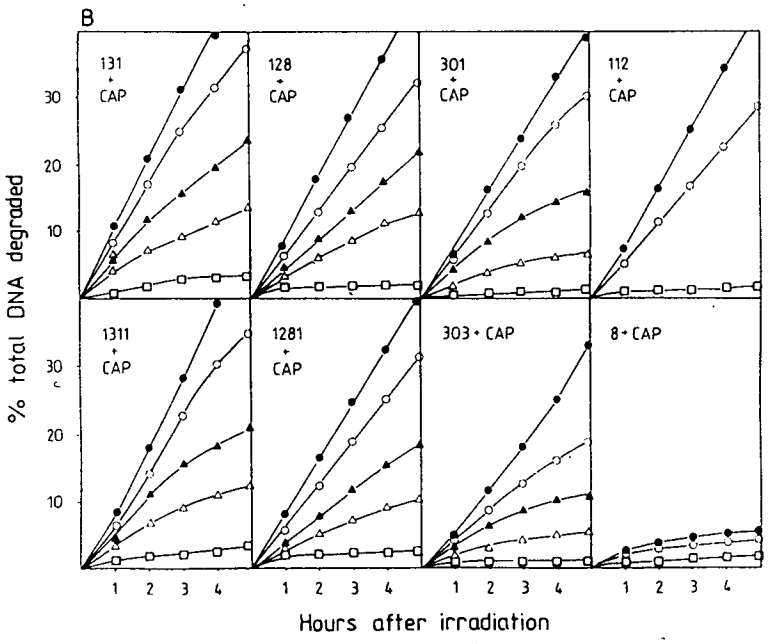
Figure 26

Release of radioactivity from ^{3}H labelled DNA into the growth medium by the MMS-sensitive *D.radiodurans* strains after UV irradiation.

- □ no UV
- Δ 15 Jm^{-2} UV
- ▲ 30 Jm^{-2} UV
- o 60 Jm^{-2} UV
- ● 125 Jm^{-2} UV

CAP indicates chloramphenicol (15μgml^{-1}) in the post-irradiation incubation medium.


A
131
128
301
112
1311
1281
303
8
% total DNA degraded
Hours after irradiation


B
131 + CAP
128 + CAP
301 + CAP
112 + CAP
1311 + CAP
1281 + CAP
303 + CAP
8 + CAP
% total DNA degraded
Hours after irradiation

more than 95% of the intracellular DNA label appeared in the growth medium after 18 h incubation.

The effect of protein synthesis on UV induced DNA degradation and survival

The effect of adding chloramphenicol to a wild type strain immediately after irradiation with different UV doses is shown in Fig.24. Chloramphenicol had no effect at doses below $60Jm^{-2}$ but above this the quantity of DNA degraded was increased above that degraded in the absence of chloramphenicol until at above $250Jm^{-2}$ the rate became dose independent at approximately 5% of total DNA h^{-1}. If the addition of chloramphenicol was delayed, the rate and extent of degradation was reduced in proportion to the delay, until after 40 minutes delay the chloramphenicol had no effect (Fig.27). This chloramphenicol-enhancible DNA degradation (CEDD) was present in the MMS-resistant strains 91, 251 and 781 which carry a wild type *mtcA* allele but chloramphenicol did not markedly enhance DNA degradation in strains UVS9, UVS25, UVS78 and 302 which lack a functional *mtcA* allele (Fig.28). Chloramphenicol enhanced the rate and extent of degradation in MMS-sensitive strains 112, 128, 1281, 131, 1311, 301 and 303 although the enhancement was not as great as in the MMS-resistant strains. Strain 8 did not display CEDD (Fig.26). Addition of chloramphenicol to the post-irradiation medium of the wild type strain and strains 91, 251 and 781 caused a decrease in the surviving fraction of cells after irradiation with UV doses exceeding $300Jm^{-2}$ (Fig. 29). However chloramphenicol had no effect on the survival

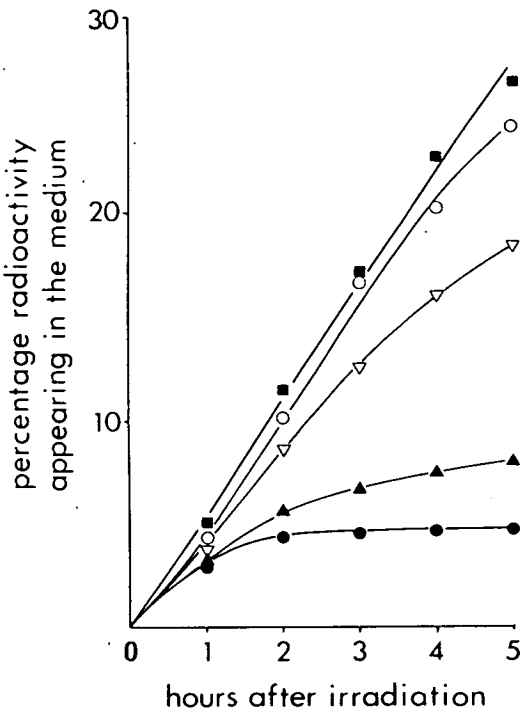


Figure 27

Release of radioactivity from ^{3}H labelled DNA into the growth medium by the *D.radiodurans* wild type strain after UV irradiation (250 Jm^{-2}) in the presence of chloramphenicol added at various times after irradiation.

Chloramphenicol (15 $\mu g ml^{-1}$) was added:-

- ■ immediately after irradiation
- o 10 min after irradiation
- Δ 20 min after irradiation
- ▲ 30 min after irradiation
- ● 40 min after irradiation

(or not added at all)

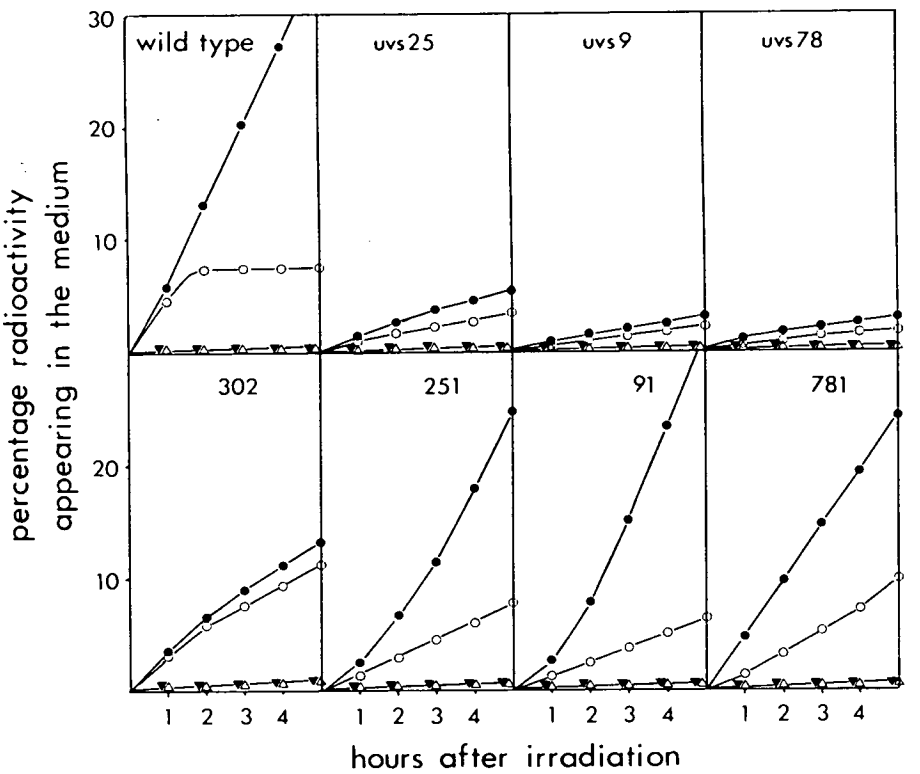


Figure 28

Release of radioactivity from ^{3}H labelled DNA into the growth medium by the MMS-resistant DNA repair-deficient *D.radiodurans* strains in the presence of chloramphenicol after UV irradiation.

- Δ no UV
- ▼ no UV plus chloramphenicol (15 μgml^{-1})
- o 500 Jm^{-2}UV
- ● 500 Jm^{-2}UV plus chloramphenicol (15 μgml^{-1})

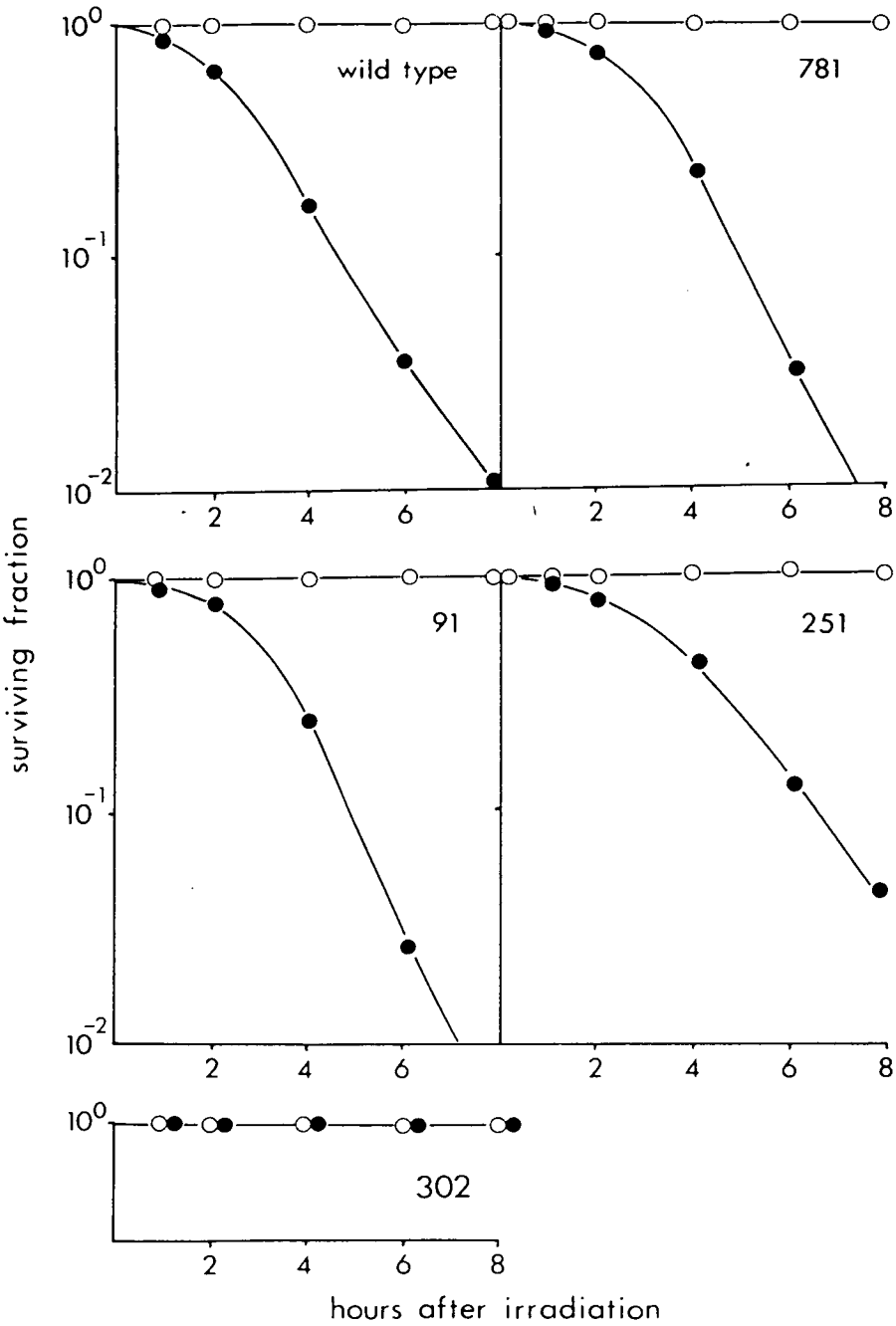


Figure 29

Effect of chloramphenicol (15 μgml^{-1}) on the survival of the *D.radiodurans* wild type strain and strains 91, 251, 302 and 781.

o no chloramphenicol

● plus chloramphenicol

of strain 302 after UV irradiation. UV-sensitive strains could not be tested for this effect because the UV doses at which it became apparent in the wild type and strain 302 exceeded their lethal doses.

Isolation of mutants resistant to chloramphenicol-enhancible lethality (CEL)

The lack of CEL (and CEDD) in strain 302 could conceivably have been due to mutation in a gene closely linked to *mtcA*. An attempt to resolve this was made by isolating strains from the wild type that survived when incubated in the presence of chloramphenicol after UV irradiation at $380Jm^{-2}$ and then to identify whether the lack of CEL in these strains was due to a mutation in the *mtcA* gene or not. Strains were isolated from the wild type by incubating UV-irradiated cultures overnight in TGY broth containing chloramphenicol. The chloramphenicol was removed by centrifugation and resuspension of the bacteria and the culture allowed to grow. This procedure was repeated. CEL^- clones were isolated by placing a sample of culture from the second selection on sterile millipore filters (0.45μm) which lay on dried TGY agar plates containing chloramphenicol ($10\mu gml^{-1}$). The filters were irradiated on the plates with $380Jm^{-2}$ and incubated overnight. The filters were transferred onto TGY plates lacking chloramphenicol and the surviving bacteria allowed to grow. The effect of the 302 mutation in the *mtcA* gene on survival in the presence of chloramphenicol using this disc method is illustrated in Fig. 30. Clones which grew on the discs were tested for chloramphenicol sensitivity ($15\mu gml^{-1}$) and sensitivity to mitomycin C

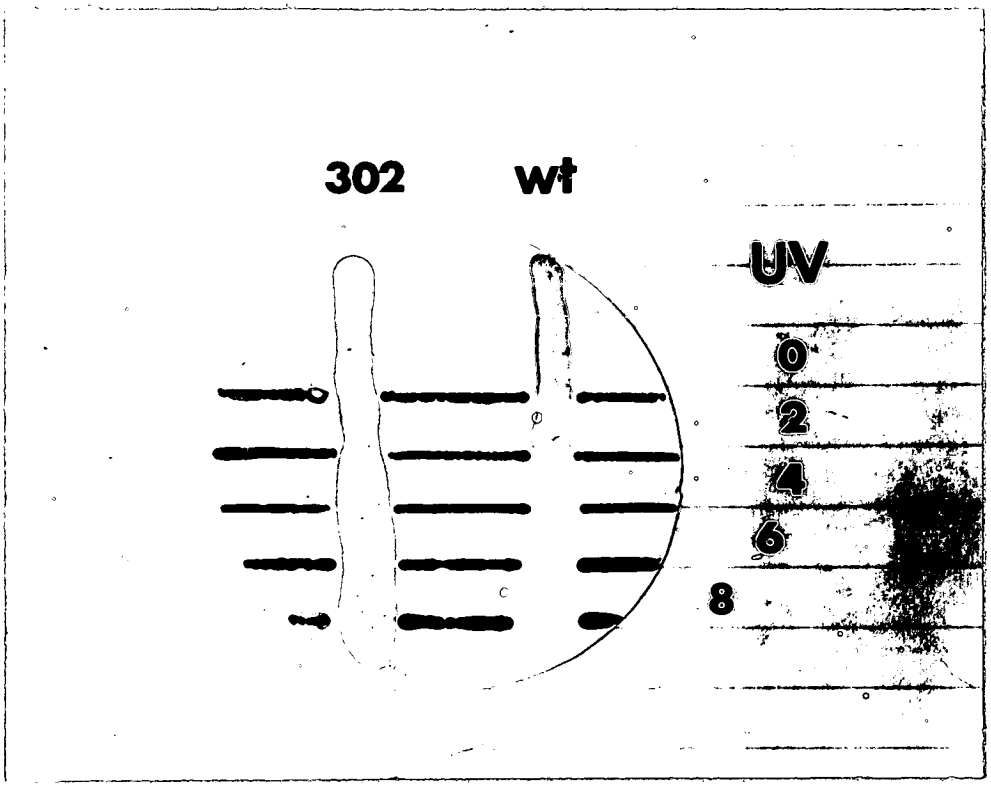


Figure 30

Demonstration of the prevention of chloramphenicol enhanced lethality by the *mtcA* mutation.

Approximately 10^6 cells in 100 µl TGY from exponentially growing cultures were streaked on a millipore filter. After absorption of the TGY the streaks were exposed to increasing UV doses. Incubation was then as described in the text. "Wt" indicates *D.radiodurans* wild type. 302 indicates strain 302 (*mtcA*$^-$). UV doses are in mins exposure and correspond to UV doses of 125 Jm^{-2} (2), 250 Jm^{-2} (4), 360 Jm^{-2} (6) and 500 Jm^{-2} (8).

($0.05\mu g ml^{-1}$) by plating on TGY agar containing these agents. Half the clones were mitomycin C sensitive but had high spontaneous reversion frequencies of approximately 10^{-4} which made transformation of these strains with DNA from strain 302 impractical. DNA was extracted from two of the mitomycin C-sensitive strains and used to transform strain 302. DNA from one of these strains failed to transform strain 302 indicating that it contained a mutation in the *mtcA* gene.

Effect of EDTA, phosphate buffer and caffeine on UV-induced DNA degradation

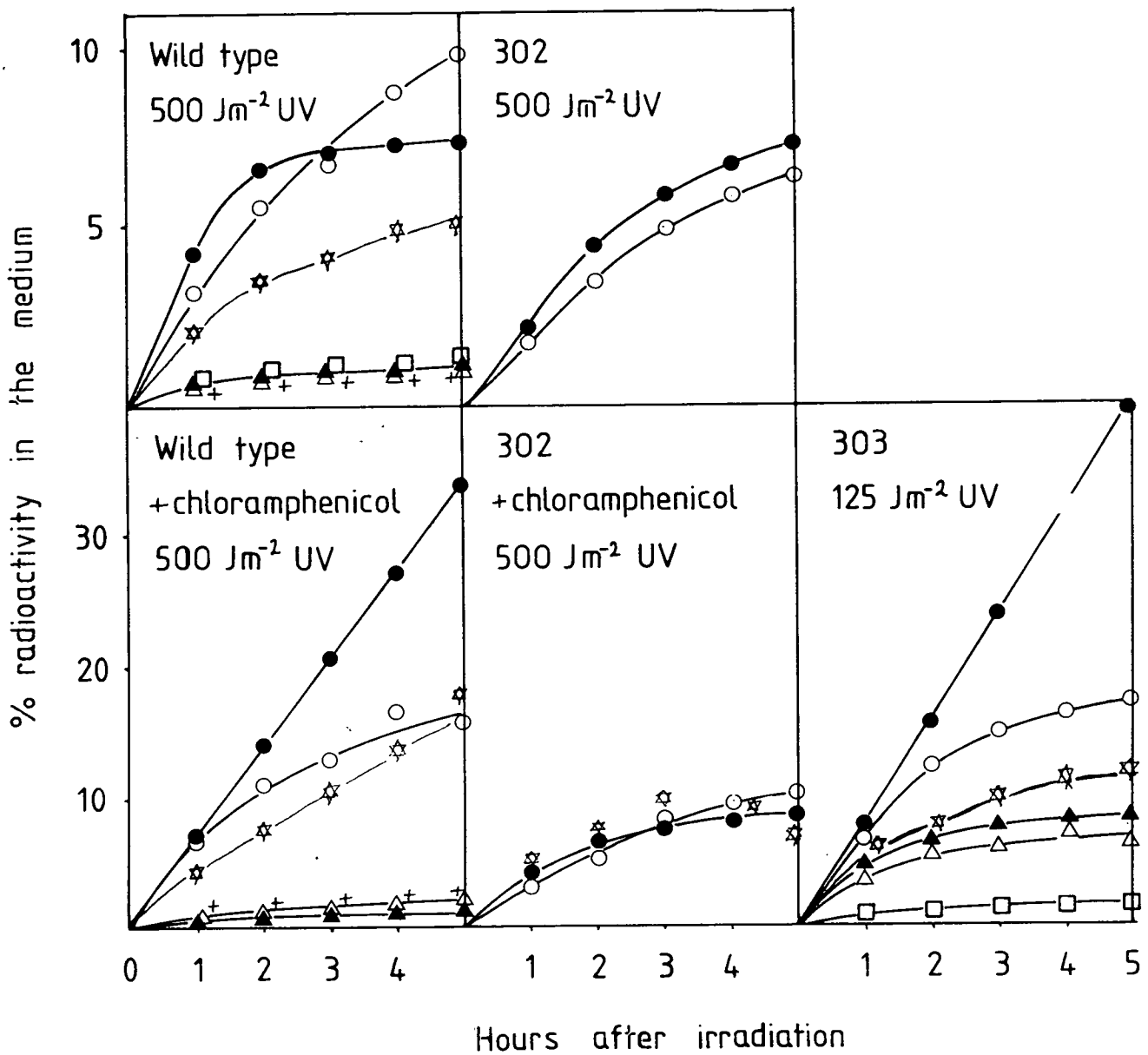
The presence of EDTA (20mM) in the growth medium after UV irradiation prevented DNA degradation in the wild type strains (Fig.31), whereas caffeine ($2.5mg ml^{-1}$) had no effect (data not shown). Degradation was also prevented if the bacteria remained in 67mM phosphate buffer after irradiation which is consistent with the absence of pyrimidine dimer excision in phosphate buffer (Boyle and Setlow 1966) (Fig.32). The addition of chloramphenicol failed to enhance degradation in the presence of EDTA or when bacteria were held in phosphate buffer (Fig.31). Since degradation was inhibited by the presence of EDTA, an excess of various divalent cations (5mM excess) was added in an attempt to identify the preferred divalent cation of the nucleases responsible for the degradation. $MnCl_2$ restored degradation in the wild type strain, whereas $MgCl_2$ or $CaCl_2$ did not. However, the rate and extent of degradation after addition of Mn^{2+} was different from the control rate of degradation (Fig.31). Mn^{2+} ions also restored the EDTA-inhibited degradation in strain 302. EDTA also inhibited the excessive UV-induced DNA degradation observed

Figure 31

Effect of EDTA (20mM) and the subsequent addition of divalent cations (25mM) on the release of radioactivity from ^{3}H labelled DNA into the growth medium by the *D.radiodurans* wild type strain and strains 302 and 303.

Incubation after UV irradiation in:-

- ● TGY
- □ TGY plus EDTA
- Δ TGY plus EDTA plus $CaCl_2$
- ▲ TGY plus EDTA plus $MgCl_2$
- o TGY plus EDTA plus $MnCl_2$
- \+ phosphate buffer
- * TGY plus EDTA plus $MnCl_2$ without UV


Wild type
500 Jm-2 UV
302
500 Jm-2 UV
Wild type
+chloramphenicol
500 Jm-2 UV
302
+chloramphenicol
500 Jm-2 UV
303
125 Jm-2 UV
% radioactivity in the medium
Hours after irradiation
10
5
30
20
10
0
1
2
3
4
5

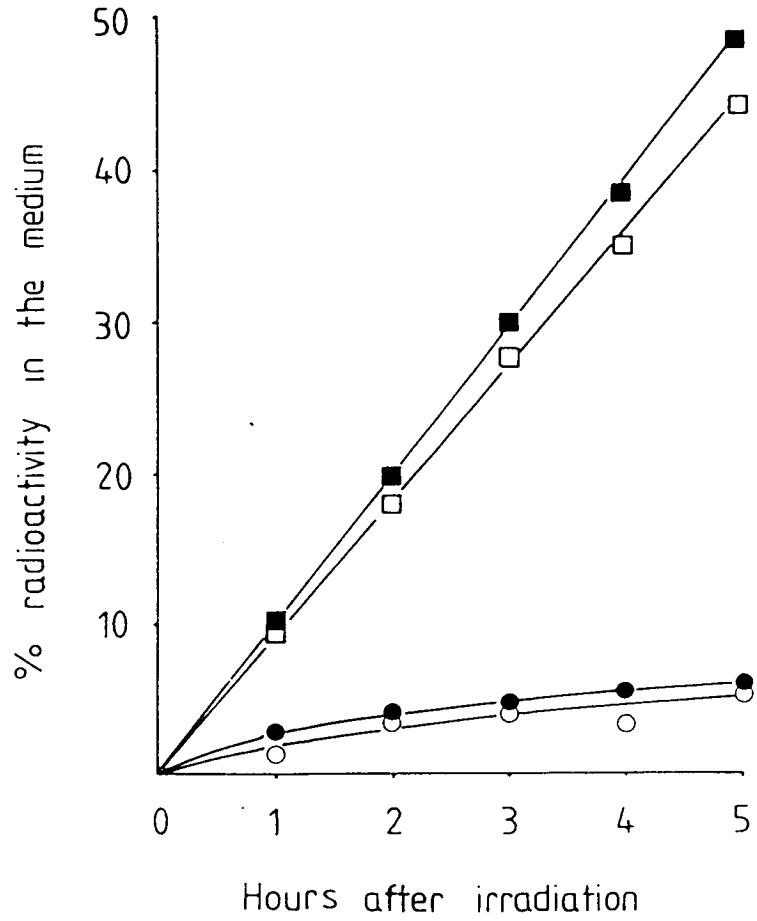


Figure 32

Effect of 67mM phosphate buffer on the release of radioactivity from ^{3}H labelled DNA into the growth medium after UV irradiation of strains 301 and 303.

■ 301 in TGY medium

□ 303 in TGY medium

● 301 in phosphate buffer

o 303 in phosphate buffer

in MMS-sensitive strains (represented by strain 303) and this could also be partially restored by the addition of divalent cations, particularly Mn^{2+} (Fig.31). The excessive degradation was also eliminated in strains 301 and 303 by incubating them in phosphate buffer after UV irradiation (Fig.32).

Effect of EDTA and divalent cations on UV survival

Since EDTA prevented the enhanced DNA degradation produced in the presence of chloramphenicol, the effect of EDTA on chloramphenicol-enhanced lethality was measured. EDTA or the presence of Ca^{2+} or Mg^{2+} ions plus chloramphenicol in the growth medium had no effect on survival of the wild-type strain unless the bacteria were UV irradiated. However Mn^{2+} ions promoted cell death in the absence of UV, the effect being exaggerated by UV irradiation (Fig.33).

DNA degradation after exposure to mitomycin C or MMS

Treatment of the wild-type strain or strain 781 with increasing doses of mitomycin C produced a similar dose-dependent amount and dose-independent rate of DNA degradation to that observed after UV irradiation. However, in strains 302 and 78 both the amount and rate of DNA degradation was very much reduced relative to the wild-type strain and strain 781 (Fig. 34). Prevention of protein synthesis after exposure to mitomycin C also promoted excessive DNA degradation in the wild type and strain 781 in a similar manner to that observed after UV irradiation but not in strains 78 and 302 in which the *mtcA* gene is mutant (Fig.34). The MMS-sensitive strains 301, 303, 131 and 1311 degraded an excess of DNA after exposure to MMS but did not degrade an excess even in the presence of a functional *mtcA* allele in strains 301 and 1311 (Fig.35).

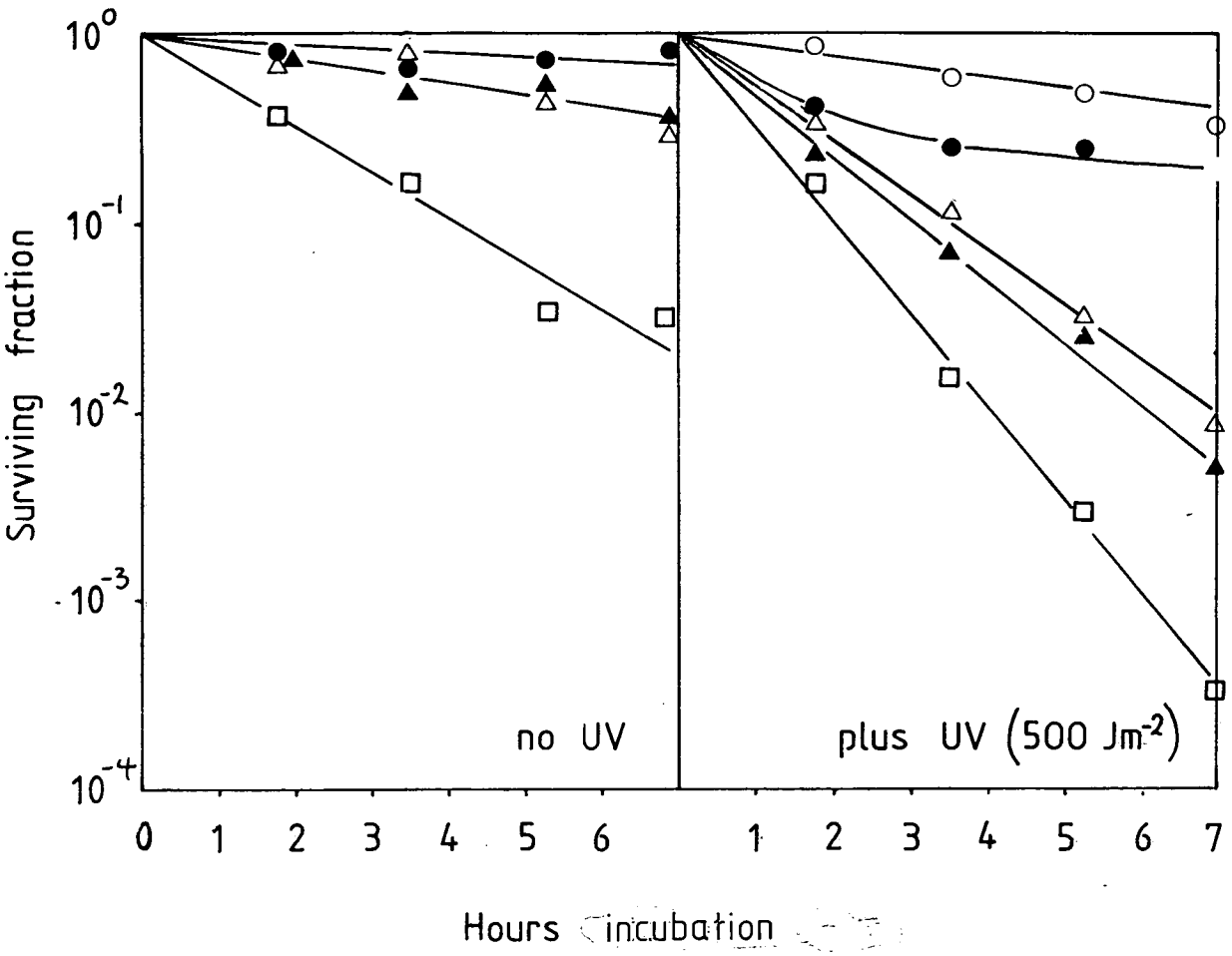


Figure 33

Effect of EDTA (20mM) and the subsequent addition of divalent cations on the survival of *D.radiodurans* wild type strain after UV irradiation during incubation in growth medium containing chloramphenicol (15 $\mu g ml^{-1}$).

- o no additions
- ● plus EDTA
- Δ plus EDTA plus $CaCl_2$ (25mM)
- ▲ plus EDTA plus $MgCl_2$ (25mM)
- □ plus EDTA plus $MnCl_2$ (25mM)

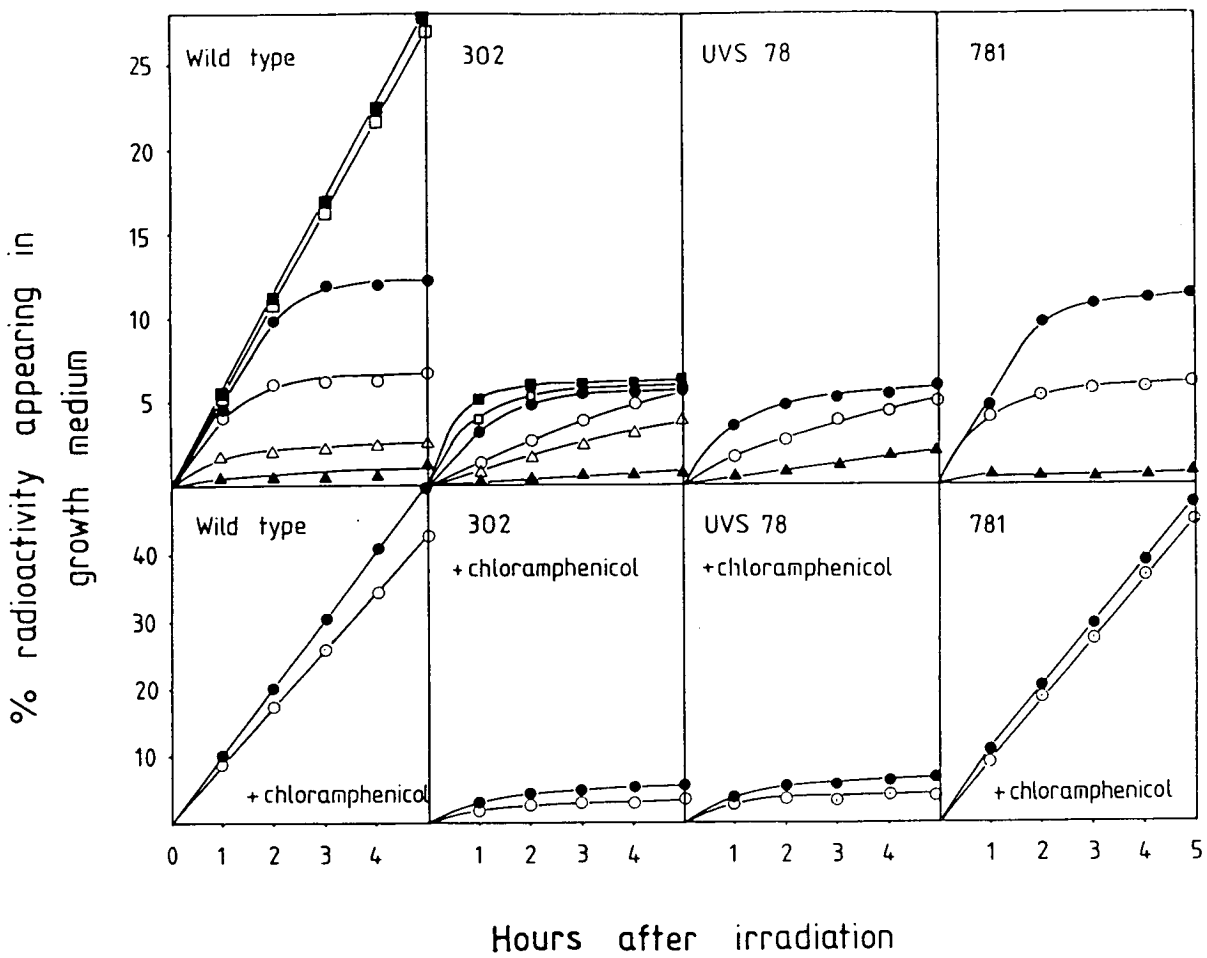


Figure 34

Release of radioactivity from ^{3}H labelled DNA into the growth medium by the wild type strain and repair-deficient *D.radiodurans* strains after exposure to mitomycin C.

▲ no mitomycin C

△ 30 $\mu g ml^{-1} min$ mitomycin C

○ 60 $\mu g ml^{-1} min$ mitomycin C

● 120 $\mu g ml^{-1} min$ "

□ 240 $\mu g ml^{-1} min$ "

■ 480 $\mu g ml^{-1} min$ "

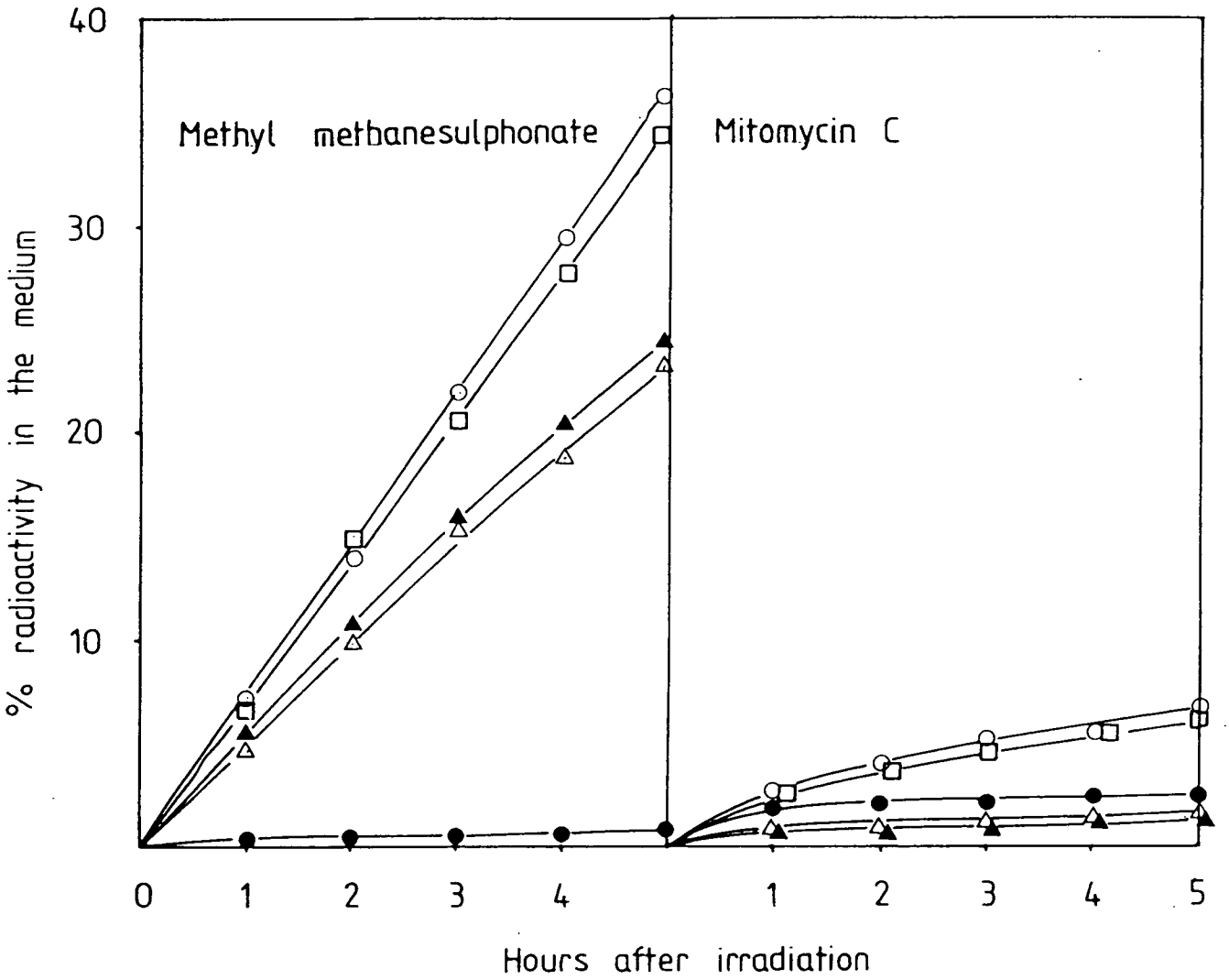


Figure 35

Amount of radioactivity released from ^{3}H labelled DNA into the growth medium by the wild type and MMS-sensitive strains of *D.radiodurans* after exposure to MMS (20mM hr) or mitomycin C (30 $\mu g ml^{-1} hr$).

- ● wild type strain
- o strain 131
- □ strain 1311
- ▲ strain 301
- △ strain 303

Part D. Recovery of cells from DNA damage

Repair of single-strand breaks produced in response to UV irradiation

Incubation of the wild-type strain in growth medium after irradiation led to a decrease in the number of single-strand breaks. All breaks produced after $125Jm^{-2}$ of UV were restored within 2h (Fig.36). In the presence or absence of post-irradiation protein synthesis, about half the breaks were repaired in 1h but protein synthesis was required for the remaining breaks to be repaired (Fig.36). The addition of EDTA (20mM) to the growth medium caused an increase in the number of single-strand breaks after irradiation (Fig.36) whereas caffeine ($2.5mgml^{-1}$) did not (data not shown). The response of strain 302 was the same as that of the wild type in repair of breaks in the presence or absence of chloramphenicol (Fig. 36). Strain 251 repaired more than 90% of breaks introduced in response to $125Jm^{-2}$ of UV within 2h of incubation but repaired fewer breaks than the wild type in the presence of chloramphenicol. Strain 781 failed to repair any breaks within 2h while strain 91 repaired less than 20%. The MMS-sensitive strains 301, 303, 131, 1311, 128 and 1281 all failed to repair any of the single-strand breaks produced in response to $20Jm^{-2}$ of UV within 2h (Fig.37). In contrast, strain 112 repaired half the single-strand breaks produced after $20Jm^{-2}$ of UV in 2h. Strain 8 responded in a manner similar to the wild type repairing all the breaks produced in response to $125Jm^{-2}$ of UV in 2h.

Figure 36

Sedimentation profiles in alkaline 5-20% sucrose gradients of DNA from the MMS-resistant incision proficient, DNA repair deficient *D.radiodurans* strains after UV irradiation (125 Jm^{-2}) and various times of post-UV irradiation incubation in growth medium.

- o no incubation
- ▲ 60 min incubation
- Δ 90 min incubation
- ● 120 min incubation
- □ 60 min incubation in TGY plus CAP (15 μgml^{-1})
- ■ 120 min incubation in TGY plus CAP (15 μgml^{-1})
- + 120 min incubation in TGY plus 20mM EDTA.

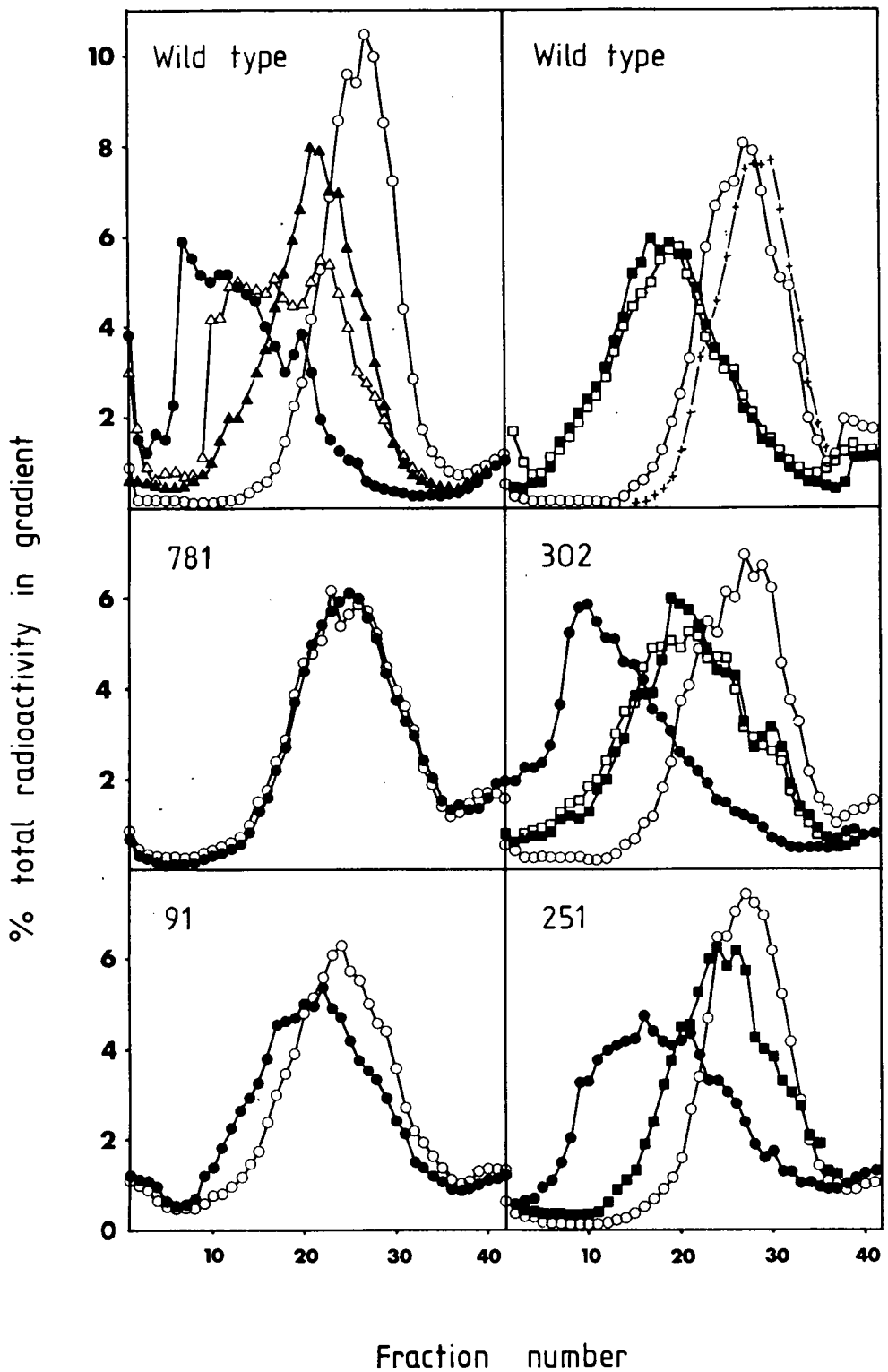
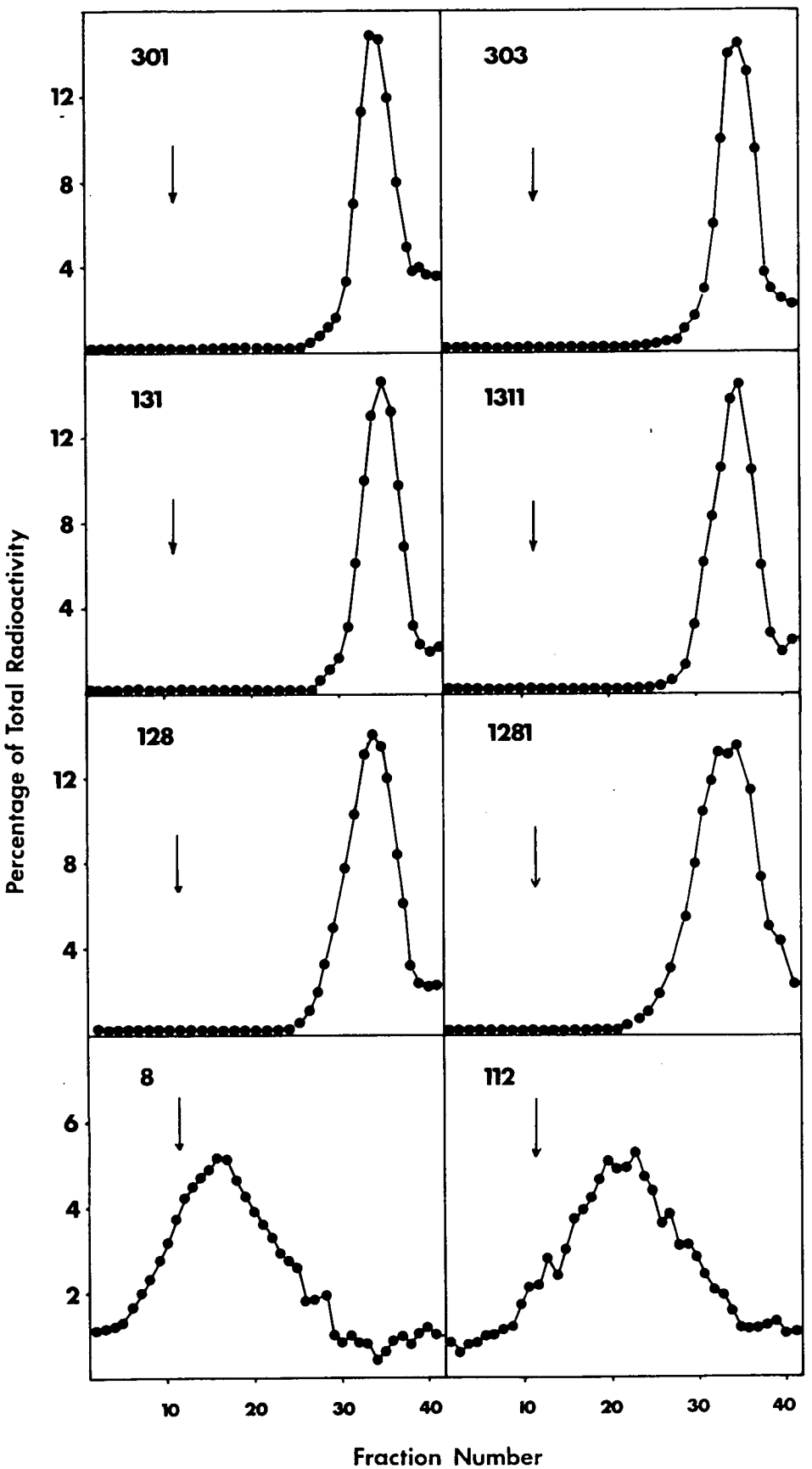

Wild type
Wild type
781
302
91
251
% total radioactivity in gradient
Fraction number
0
2
4
6
8
10
10
20
30
40

Figure 37

Sedimentation profiles in alkaline 5-20% sucrose gradients of DNA from the MMS-sensitive *D.radiodurans* strains after UV irradiation (15 Jm^{-2}) and 2 hr incubation in growth medium.


301
303
131
1311
128
1281
8
112
Percentage of Total Radioactivity
Fraction Number
12
8
4
6
2
10
20
30
40

Recovery of DNA synthesis after UV irradiation

The effects of chloramphenicol, EDTA and caffeine on DNA synthesis after UV irradiation of the wild-type strain are shown in Fig.38. All three depressed DNA synthesis in the absence of DNA damage (the maximum sublethal caffeine concentration, 2.5mg ml^{-1}, apparently completely arrested DNA synthesis), but the depression was enhanced by UV irradiation in proportion to the quantity of UV damage.

The effect of UV irradiation on DNA synthesis in the MMS-resistant strains is shown in Fig.39. The lag period introduced by irradiation of strain 302 was similar to that of the wild type strain. However the rate of synthesis was greatly reduced in strains UVS9 and UVS25 and absent in strain UVS78. The residual rate of incorporation was exponential. Strains 91, 251 and 781 showed an inhibition of synthesis which was intermediate between the wild type strain and strains UVS9, UVS25 and UVS78, with a residual rate of synthesis that was exponential above $300Jm^{-2}$.

In the MMS-sensitive strains (Fig.40) DNA synthesis was interrupted by a dose of $10Jm^{-2}$ of UV but recovered after a lag period which varied between the strains. Strain 131 produced the longest lag period. Strain 8 in contrast showed a level of inhibition of DNA synthesis comparable to that of the wild type.

Recovery of viability of bacteria after UV irradiation

The rationale for these experiments was that since a bacterium has a finite capacity to repair a certain quantity of DNA damage, if it is given time to repair some of this

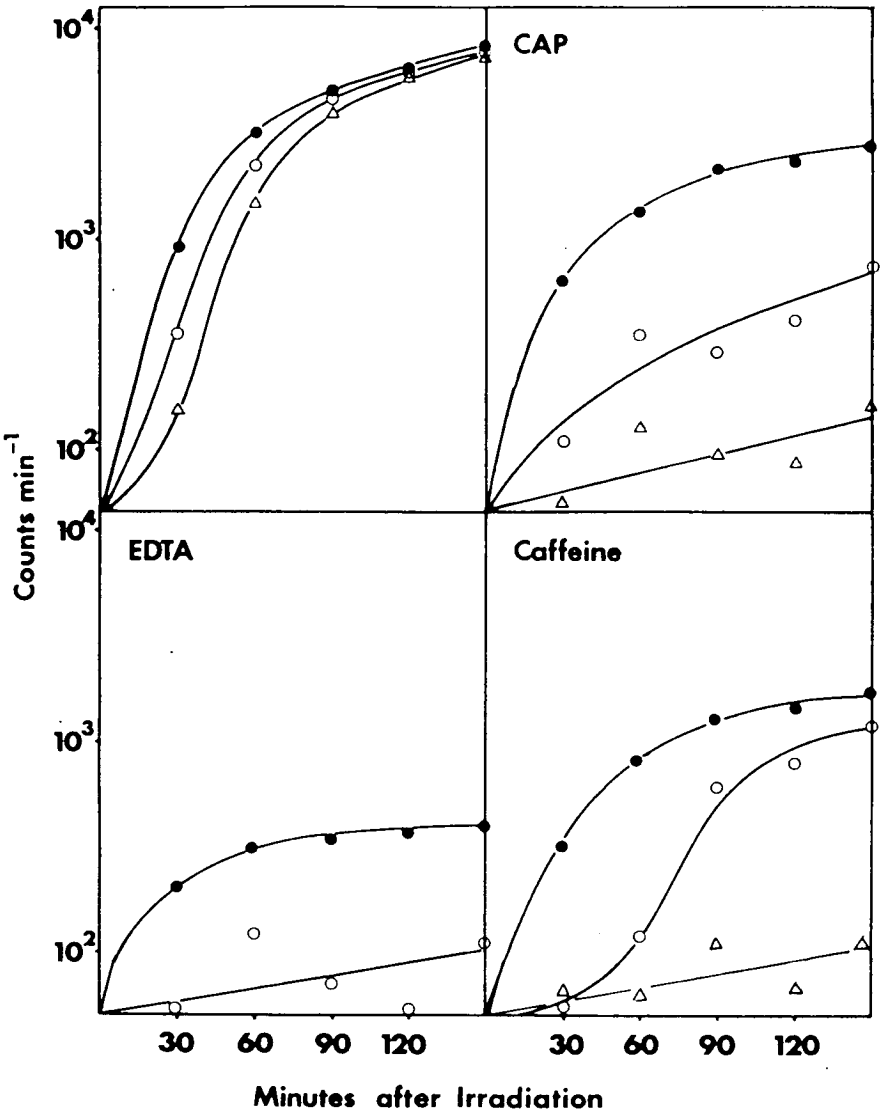


Figure 38

Uptake of ^{3}H thymidine into TCA insoluble material by the *D.radiodurans* wild type strain after UV irradiation in the presence of chloramphenicol (15 $\mu g ml^{-1}$), EDTA (10mM) or caffeine (1 $mg ml^{-1}$)

- ● no UV
- o 60 Jm^{-2} UV
- Δ 125 Jm^{-2} UV

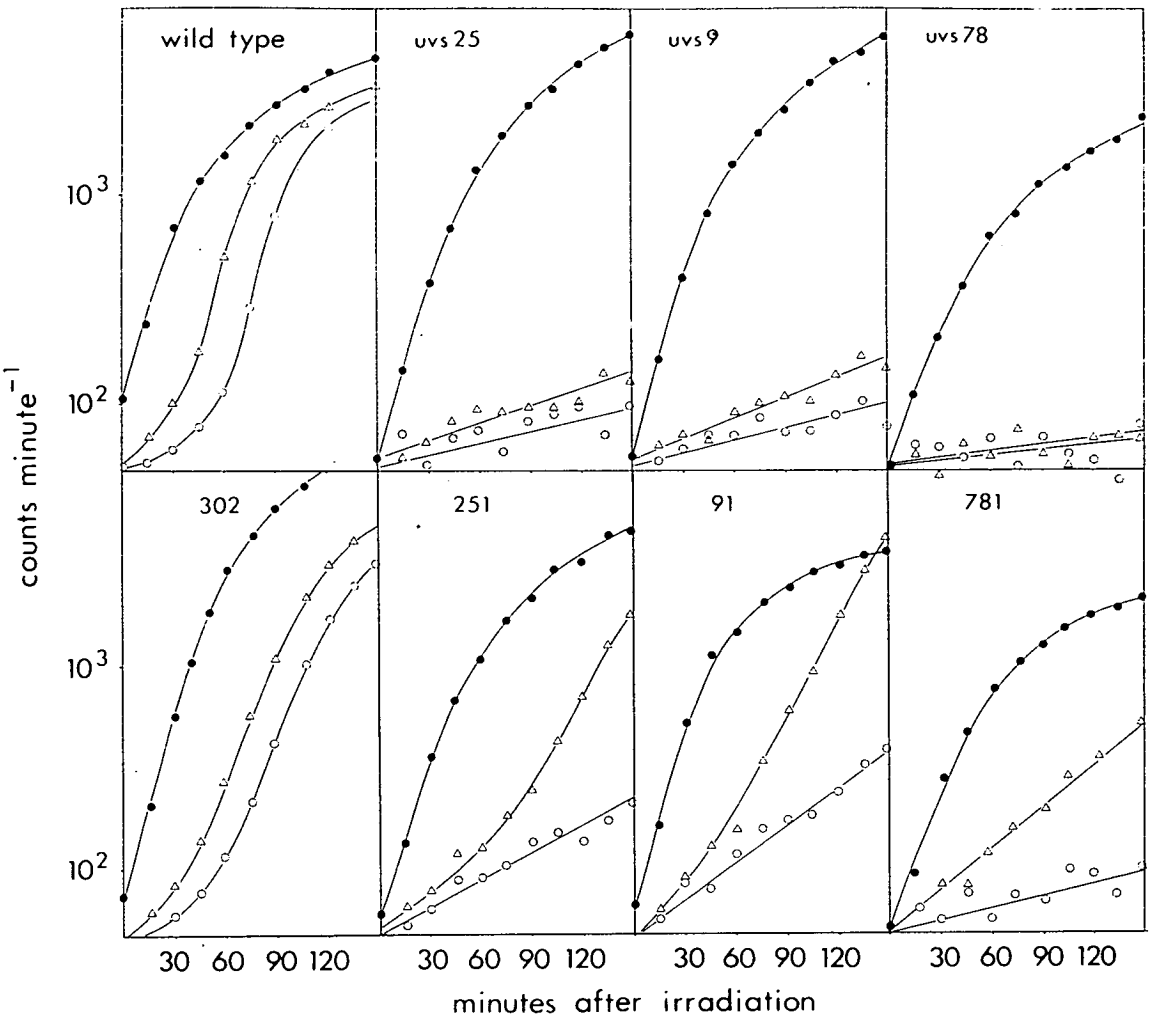


Figure 39

Uptake of ^{3}H-thymidine into TCA-insoluble material by the MMS-resistant, DNA repair-deficient strains of *D.radiodurans* after UV irradiation.

● no UV

Δ 180 Jm^{-2}

○ 360 Jm^{-2}

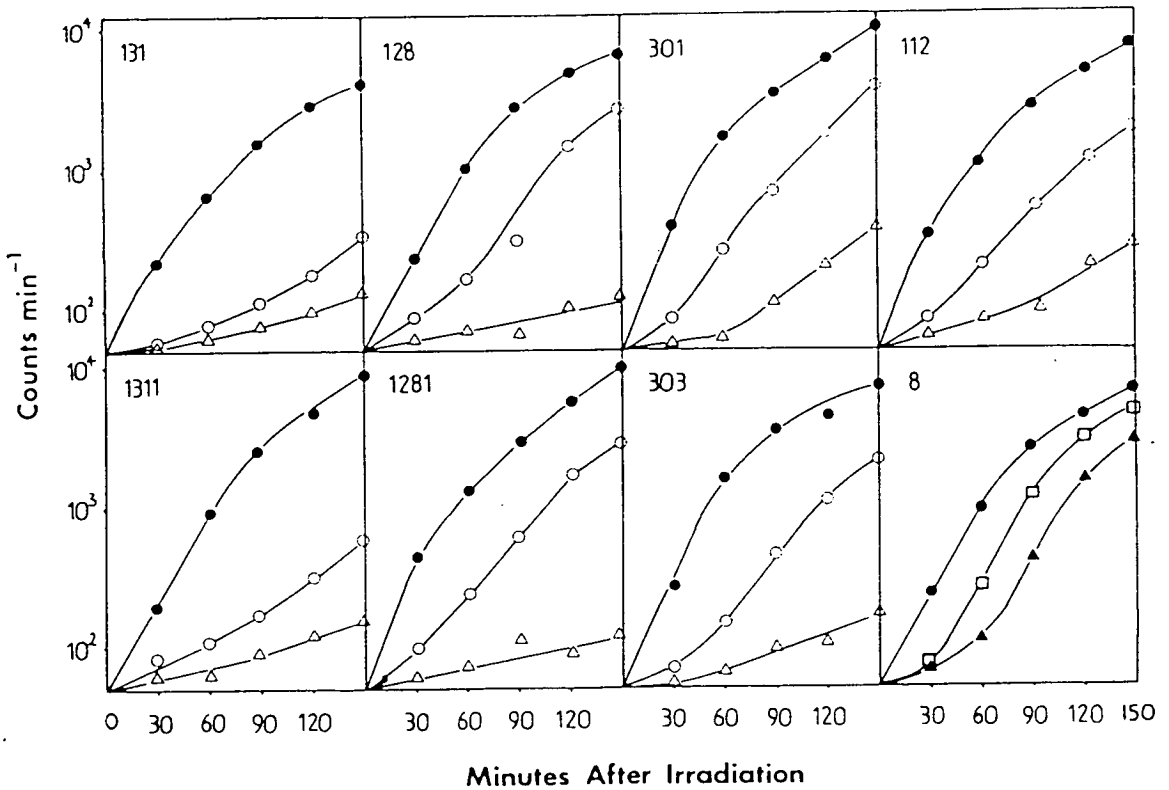


Figure 40

Uptake of ^{3}H-thymidine into TCA-insoluble material by MMS-sensitive strains of *D.radiodurans* after UV irradiation.

- ● no UV
- o 10 Jm^{-2} UV
- Δ 30 Jm^{-2} UV
- □ 180 Jm^{-2} UV
- ▲ 360 Jm^{-2} UV

damage then the capacity of the bacterium to survive additional DNA damage is increased. Bacteria that are slower than the wild type in completing DNA repair can therefore be distinguished since they will retain more DNA damage after a certain period allowed for repair and this will lead to a relatively lower survival after a second exposure to DNA damage. The initial UV dose given allowed almost 100% survival being just at the end of the shoulder of the survival curve. A second, equal UV dose was given immediately or after a period of incubation in TGY medium and the surviving fraction of bacteria determined.

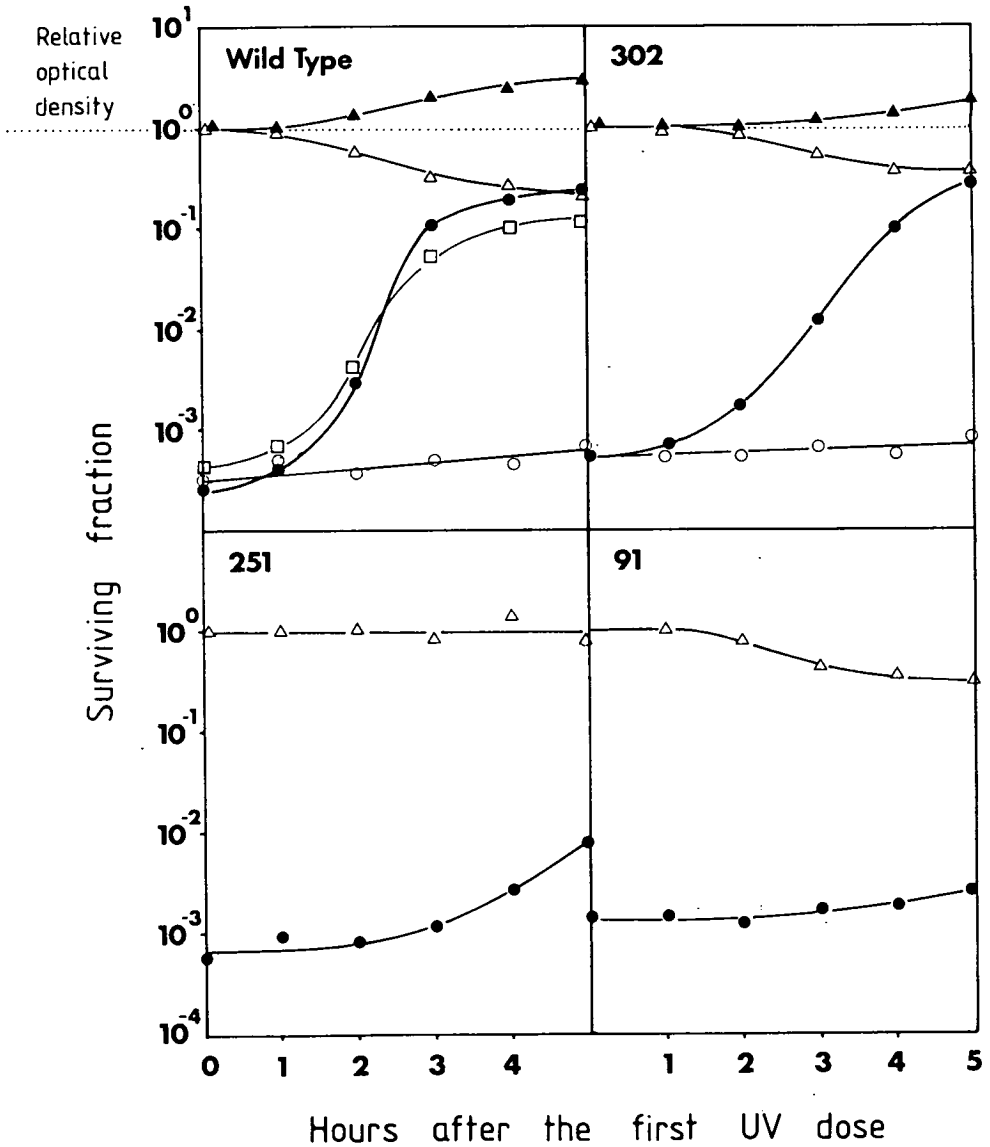
The wild type strain showed no recovery of viability during the first hour after irradiation with the first UV dose but recovered viability maximally within the next two hours (Fig.41). The recovery was prevented by the presence of EDTA (20mM) or chloramphenicol ($15\mu g ml^{-1}$) in the growth medium but not by caffeine ($2.5mg\ ml^{-1}$). Unexpectedly, the survival of the control culture receiving only the first UV dose showed a gradual decline in viability with time whilst the optical density of the culture was rising. A similar pattern of events to those occurring in the wild type was seen in strain 302 (Fig.41). However, strains 251 and 91 showed virtually no recovery from the first UV dose in 5 hours. Strain 91 also showed a decline in viability after a single UV dose and continued incubation whereas strain 251 did not. Strain 781 was not compared directly with the other strains because of its greater sensitivity to UV irradiation. However, its recovery from a dose of $450Jm^{-2}$ of UV is shown in Fig.42 and shows an exponential rate of recovery unlike the other strains ie. it lacks a lag period. There is also a decline in viability

Figure 41

Sensitivity to a second UV dose of a UV irradiated culture of *D.radiodurans* wild type strain and strains 302, 91 and 251 after incubation in growth medium for various times.

First and second UV doses were 550 Jm^{-2}

- ▲ relative optical density of the culture
- Δ culture receiving first UV dose only
- ● culture receiving both UV doses
- o as ● plus chloramphenicol (15 μgml^{-1}) in incubation medium
- □ as ● plus caffeine (2.5 $mgml^{-1}$) in incubation medium


Relative optical density
Wild Type
302
251
91
Surviving fraction
Hours after the first UV dose

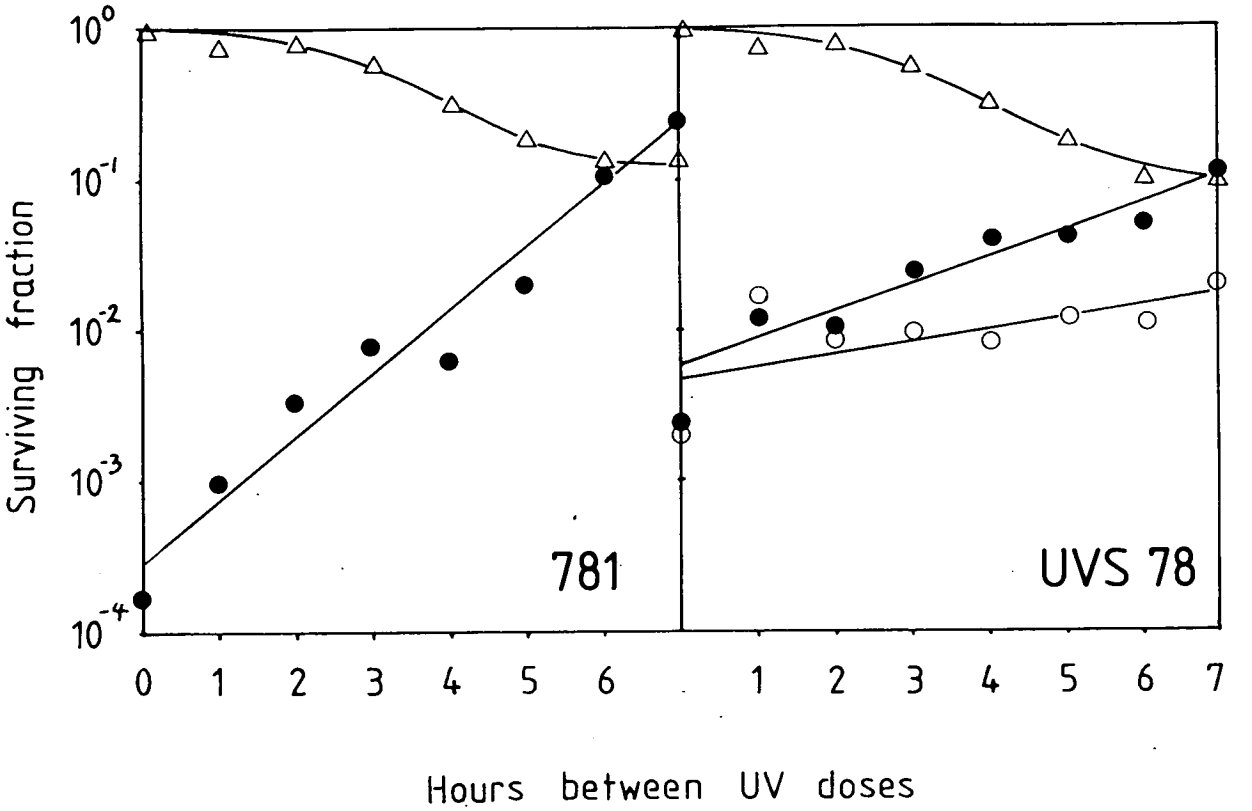


Figure 42

Sensitivity to a second UV dose of UV irradiated cultures of *D.radiodurans* strains 781 and UVS78 after incubation in TGY medium for various times.

First and second UV doses:- 300 Jm^{-2} for 781,
40 Jm^{-2} for UVS78

△ culture receiving first UV dose only

● culture receiving both UV doses

o as ● plus chloramphenicol (15 μgml^{-1}) in incubation medium

after a single UV dose and continued incubation.

Strain UVS78 is excision-repair deficient, yet is still as UV resistant as *E.coli* wild-type strains indicating the presence of further repair mechanisms for repair of UV damage. The speed at which strain 78 recovers viability was therefore measured to determine the speed of action of these other processes. Recovery from $40Jm^{-2}$ UV occurred fastest within 2h after irradiation then recovered at an exponential rate to control levels over the next 5h. This process required protein synthesis since chloramphenicol prevented the recovery (Fig. 42).

The influence of excision repair on repair of UV irradiated transforming DNA

UV irradiation *in vitro* is known to inactivate transforming DNA in *B.subtilis* (Hadden 1981), *Haemophilus influenza* (Setlow *et al.*, 1968) and *D.radiodurans* (Moseley and Mattingly 1971). In the first two systems transforming DNA can be repaired by excision or postreplication repair such that mutants deficient in either express markers on UV-irradiated transforming DNA at a lower frequency than repair proficient strains (Setlow 1977). The greater reduction is observed in excision-deficient mutants (Hadden 1981). An experiment was carried out to determine the ability of the excision repair-deficient strain UVS78 to express a rifampicin-resistance marker on UV-irradiated transforming DNA. Preliminary experiments established a maximum dose of UV ($950Jm^{-2}$) for irradiation of the transforming DNA above which the transformation frequency of a recipient wild type culture to rifampicin resistance was decreased.

DNA at various dilutions, either irradiated or unirradiated was then diluted and used to transform either the wild type, strain UVS78, strains 781 or strain 302. Although the UV irradiation decreased the number of rifampicin-resistant transformants in UVS78 in a manner independent of DNA concentration, the control incision repair-proficient strains 302 and 781 showed a similar (or greater!) decrease (Table 13).

A note on measurement of recombinational exchanges in *D.radiodurans* in alkaline sucrose gradients

Excision repair-deficient *D.radiodurans* strains probably possess a functional "recombination repair" capacity which should be revealed by experiments similar to those performed by Rupp and Howard-Flanders (1968) whereby a pulse of labelled DNA precursor (^{3}H thymidine) becomes incorporated into short DNA fragments in UV irradiated *E.coli uvrA*$^{-}$ strains. The short fragments then become part of larger molecules as a result of recombinational exchanges (see introduction). However, a prerequisite for such experiments is that a control, unirradiated, wild-type culture of *D.radiodurans* should incorporate a pulse of label in a similar manner to a wild type *E.coli*. Label would be expected to stay as low molecular weight material for a short period (under 2 min) before becoming high molecular weight as a result of the incorporation of label initially into Okazaki fragments which quickly become linked to the chromosome as a whole. The proportion of radioactivity remaining low molecular weight would quickly become insignificant relative to the labelled chromosome. Initial attempts to pulse label *D.radiodurans* wild type however showed that label remained in

Table 13

Effect of UV (940 Jm^{-2}) irradiating transforming DNA on the frequency of transformation of DNA repair-deficient strains of *D.radiodurans* to rifampicin resistance.

Transforming DNA was obtained from a rifampicin resistant derivative of the wild type strain.

Strain	DNA Concentration	Frequency of Rifampicin-Resistant Transformants		Irrad./Unirrad.
Wild Type	1μg.ml^{-1}	Unirrad.	1.0×10^{-4}	3.2
		Irrad.	3.2×10^{-4}	
	10μg.ml^{-1}	Unirrad.	5.7×10^{-4}	0.8
		Irrad.	4.6×10^{-4}	
	50μg.ml^{-1}	Unirrad.	1.8×10^{-3}	2.4
		Irrad.	4.3×10^{-3}	
UVS78	1μg.ml^{-1}	unirrad.	4.2×10^{-4}	4.5×10^{-2}
		Irrad.	1.9×10^{-5}	
	10μg.ml^{-1}	Unirrad.	6.1×10^{-4}	5.7×10^{-2}
		Irrad.	3.5×10^{-5}	
	50μg.ml^{-1}	Unirrad.	1.2×10^{-3}	7.7×10^{-2}
		Irrad.	9.2×10^{-5}	
302	10μg.ml^{-1}	Unirrad.	5.2×10^{-4}	1.8×10^{-2}
		Irrad.	9.8×10^{-6}	
781	10μg.ml^{-1}	Unirrad.	1.5×10^{-4}	8.0×10^{-3}
		Irrad.	1.2×10^{-6}	

a range of low molecular weight fragments for extended periods (Fig.43). Other workers have reported a similar anomalous incorporation of label into *D.radiodurans* DNA (Moseley *et al.*, pers.comm.) which cannot be explained conveniently. In view of the unusual incorporation in the wild type it is impossible to demonstrate recombinational exchanges in *D.radiodurans* using this method.

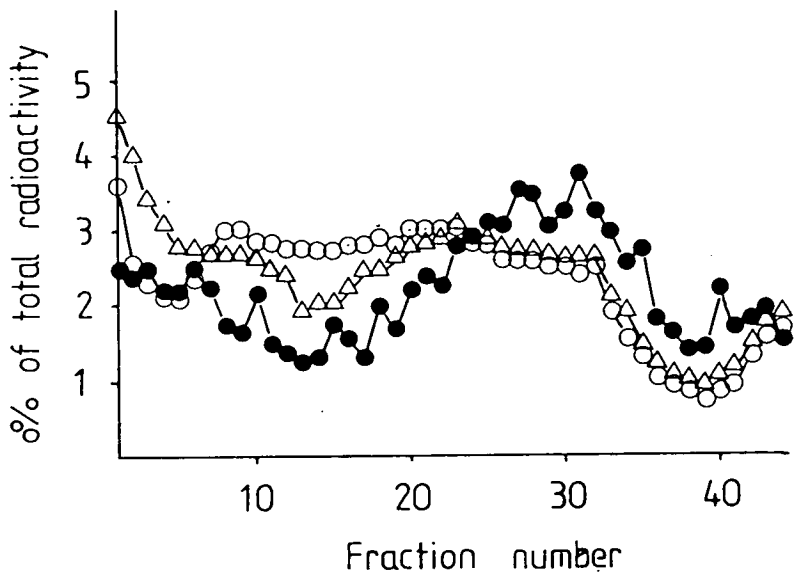


Figure 43

Sedimentation profiles in alkaline 5-20% sucrose gradients of DNA from *D.radiodurans* wild type strain incubated in growth medium containing ^{3}H-thymidine.

- ● 30 min incubation in label
- o 45 min incubation in label
- Δ 60 min incubation in label

Chapter 3

Section 2: Identification of DNA repair enzymes *in vitro*

Non-specific endonucleases and exonucleases attack undamaged DNA and therefore interfere with attempts to identify DNA damage-specific activities by destroying the substrate DNA. Initial attempts to identify DNA damage-specific endonucleases in *D.radiodurans* were frustrated by the presence of large amounts of non-specific nuclease activity. The source and nature of this activity was investigated.

Non-specific extracellular nucleases

All *D.radiodurans* strains, repair-proficient and deficient excreted large quantities of non-specific activity towards double-stranded linear salmon sperm DNA when this was included as an overlay on agar plates (Fig.44A). The other *Deinococcus* spp. also produced extracellular nucleases but to a lesser extent. The activity was also produced in broth cultures so that drops of cell-free growth medium from exponential cultures produced zones of clearing in TGY plates overlaid with salmon sperm DNA, but not if the TGY contained 20mM EDTA. The dependence of the non-specific nuclease activity on divalent cations was also demonstrated by the degradation of tritiated *D.radiodurans* DNA which failed to be retained on filter paper discs (Whatman no.1) after TCA precipitation (Fig.45). No degradation of native DNA occurred in the presence of EDTA and the degradation could be restored by the addition of Mg^{2+}, Mn^{2+} or Ca^{2+} ions with Mn^{2+} ions producing the greatest stimulation. The activity was enhanced on heat-denatured (single stranded) DNA in the presence of Mg^{2+} ions

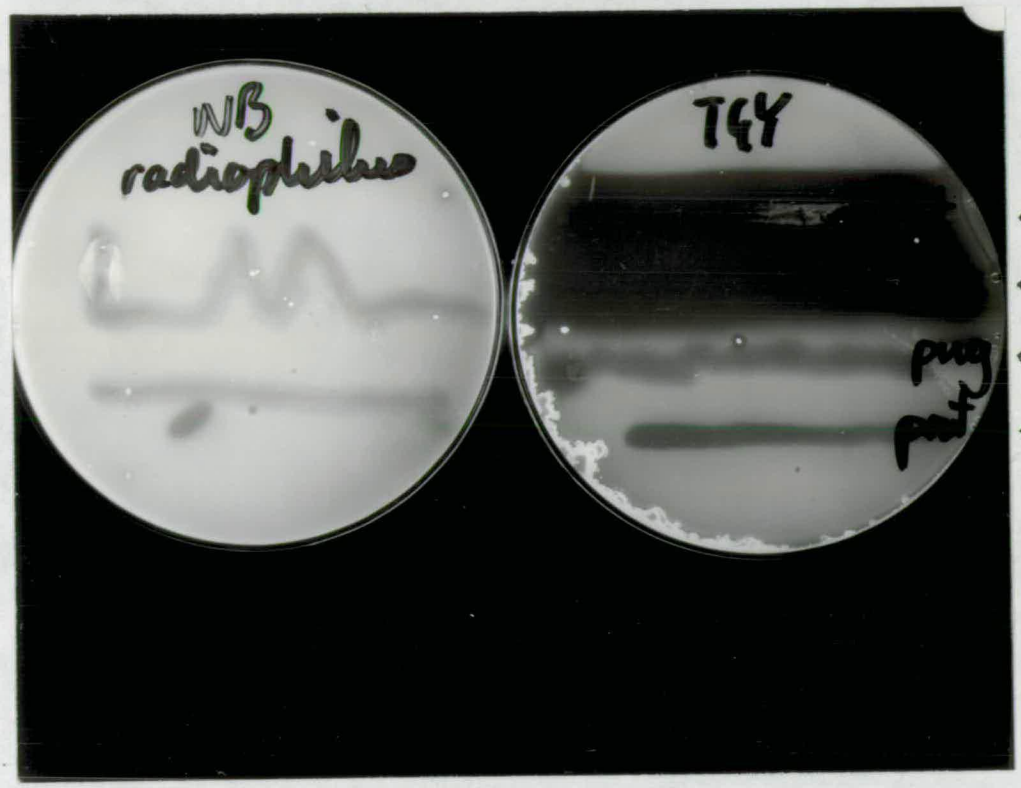
Figure 44 Non specific extracellular DNase activity

A. Degradation of native salmon sperm DNA.
Single streaks of *D.radiodurans* R, (labelled "wt") and strain sark, *D.proteolyticus* (labelled "prot"), *D.radiopugnans* (labelled "pug") and *D.radiophilus* were applied to a TGY plate (or nutrient agar in the case of *D.radiophilus*) which had been overlaid with agar (5ml) containing salmon sperm DNA (1 mg ml^{-1}). After 18 hr growth which produced comparably thick streaks, the bacteria were removed and the DNA visualised with Ethidium bromide. The zones of clearing produced by the two *D.radiodurans* strains have coalesced.

B. Non-specific conversion of covalently closed circular (ccc) PM2 DNA to the open circular form and degradation of PM2 DNA by extracellular DNase activity of *D.radiodurans*.

Divalent Cations (5mM) or EDTA (5mM)	Undamaged DNA Lanes	UV irradiated ($90Jm^{-2}$) DNA lanes
none	1 (no DNase added)	7 (no DNase added)
none	2	8
Mg^{2+}	3	9
Ca^{2+}	4	10
Mn^{2+}	5	11
EDTA	6	12

A

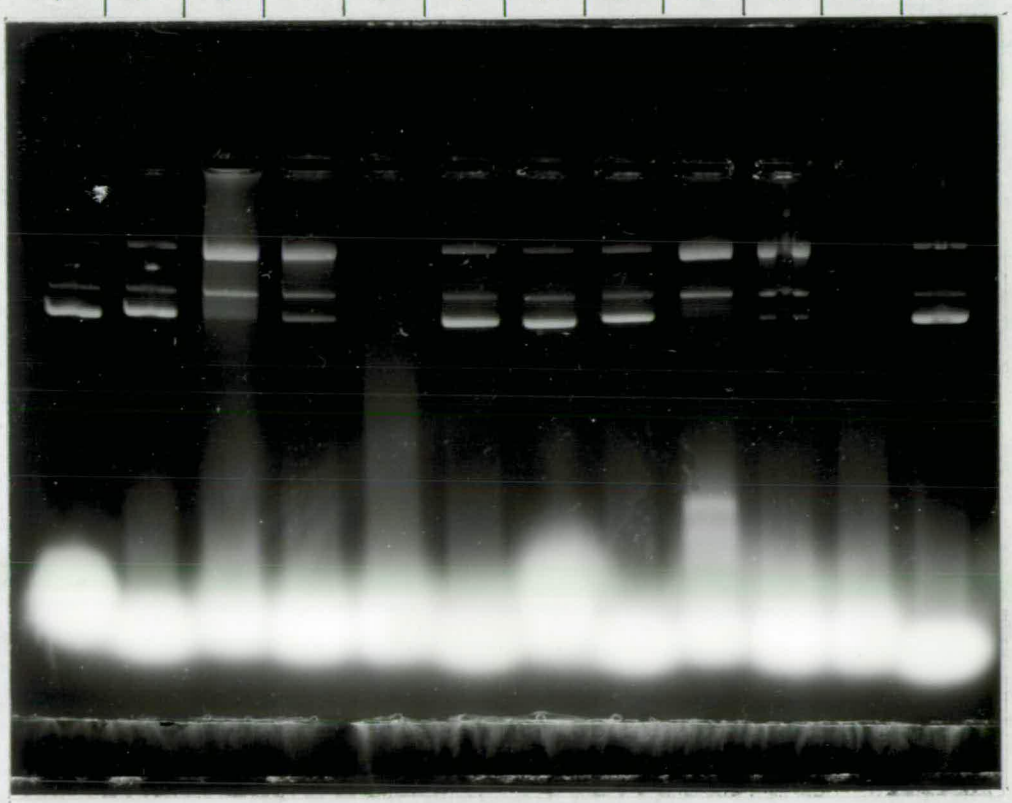


Position of bacterial strains on TGY plate

Gel lane numbers

1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12

B



oc DNA
linear DNA
ccc DNA

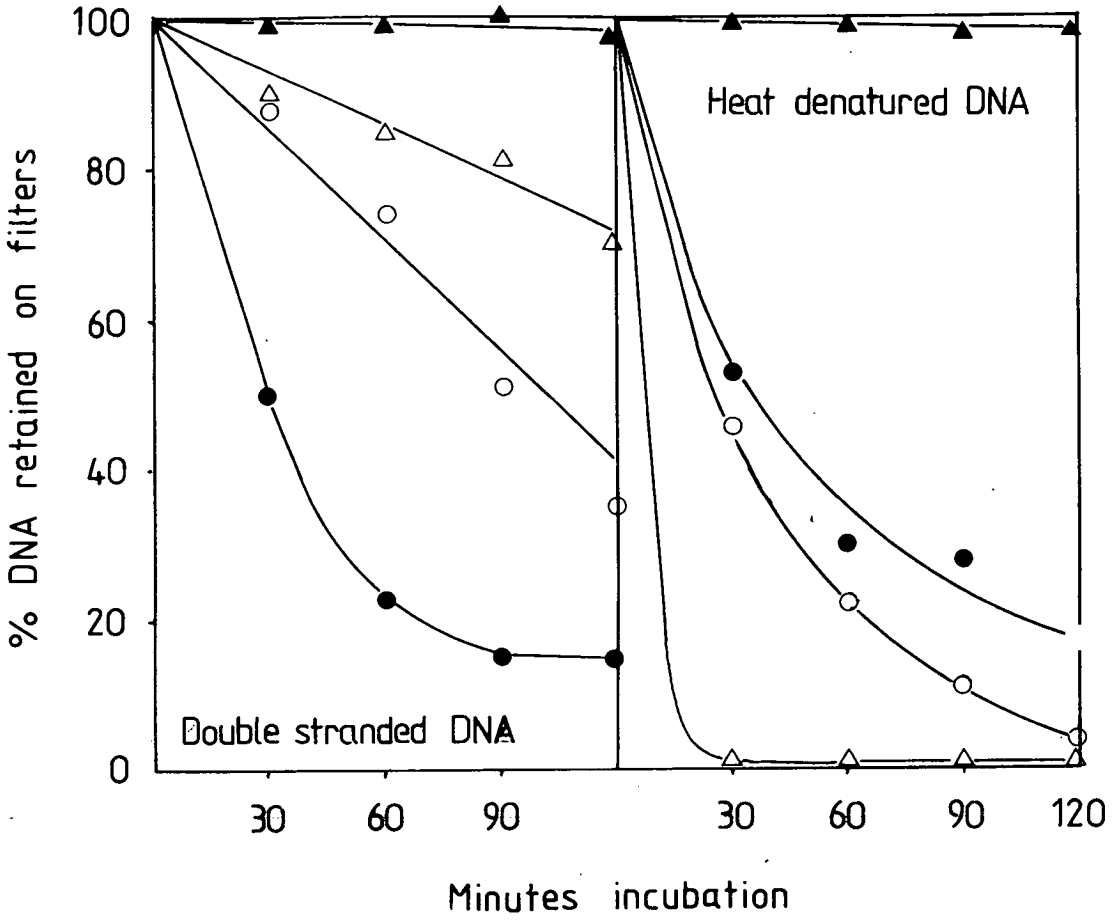


Figure 45

Retention of radioactivity on filter discs after incubation of ^{3}H-labelled DNA with cell-free growth medium obtained from a stationary phase culture of *D.radiodurans* wild type strain.

Additions to reaction mixture

▲ 10mM EDTA

△ 5mM $MgCl_2$

○ 5mM $CaCl_2$

● 5mM $MnCl_2$

or to a lesser degree by Ca^{2+} ions, but not by Mn^{2+} ions which may reflect the presence of more than one enzyme. The extracellular activity also degraded ccc DNA in a cation-dependent manner indicating the presence of an endonuclease (Fig.44B). An attempt was made to reduce the amount of extracellular non-specific nuclease activity by isolating MNNG-induced mutants of *D.radiodurans* that lacked zones of cleaving on TGY plates overlaid with salmon sperm DNA. Two such strains, *nuc1* and *nuc2* were isolated. However, both retained the extracellular endonuclease activity which may therefore have been due to a separate enzyme from that producing the zones of clearing. These strains also reverted to the wild type phenotype at high frequency and because they did not aid in the search for DNA damage-specific endonucleases they were abandoned.

The extracellular DNase activity appeared to be absent from within the wild type cells (Fig.48) and could be eliminated by extensive washing of the bacteria. When the extracellular activity was run in DNA-containing polyacrilamide gels a streak of degradation was seen and not a single band. Development of the gels for shorter times showed a predominant band of degradation of approx.MW 220000. Staining of the proteins in the gel with kenacid blue revealed two narrowly separated bands at this position in the gel either or both of which could have been an extracellular nuclease protein.

Non-specific intracellular nucleases

Lysates of washed *D.radiodurans* also produced non-specific

DNase activity against linear or ccc DNA which was largely eliminated by the addition of EDTA. This activity could be separated into upwards of thirteen distinguishable bands of different molecular weights in gels containing double-stranded or heat-denatured DNA (Fig.46). Different bands appeared in the presence of different divalent cations (Figs.46 and 47) but all were active in the presence of 10mM mercaptoethanol or 25mM NaCl and were not enhanced by 5mM ATP. However, all except bands L and M were inhibited by 5mM Zn^{2+} ions.

A possible explanation for the lack of chloramphenicol-enhancible DNA degradation in strains carrying the 302 mutation was that they lacked a particular exonuclease which could participate in excision repair and so the DNase activity of strains carrying the 302 mutation (in gene *mtcA*) was examined. No differences were observed between strains in the pattern of bands produced in the presence of double-stranded DNA when compared to the wild type. However, lysates of the same strains run in a polyacrilamide gel containing denatured DNA showed reduced levels of several bands (Fig.48).

Identification of DNA damage-specific endonucleases

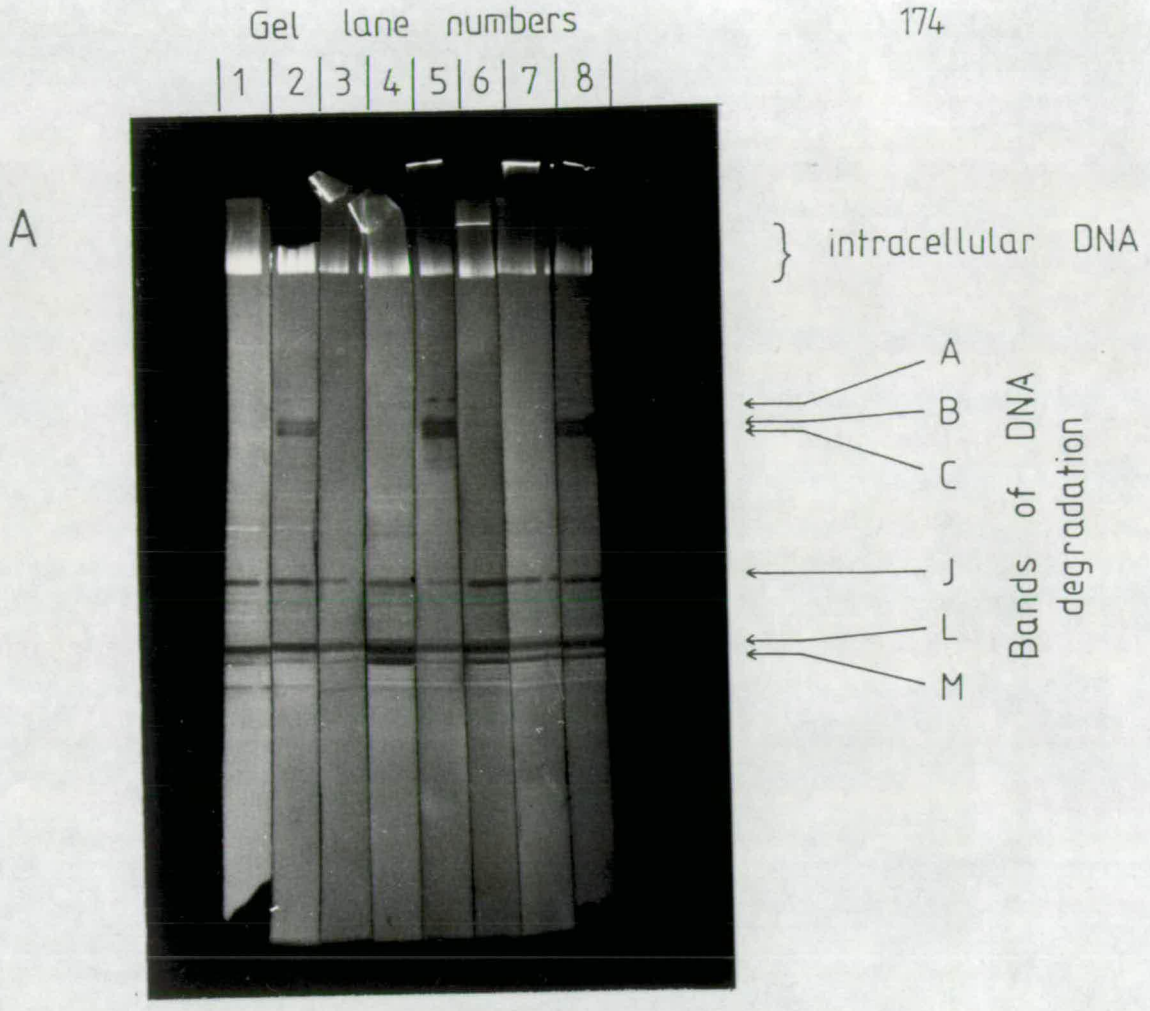
To minimise the masking of DNA damage-specific endonucleases by the non-specific enzymes, attempts were made to determine a reaction environment that would suppress intracellular non-specific DNase activity and allow DNA repair-specific activities to act. It was found that the non-specific activity was reduced in the absence of added divalent cations and was confined mainly to degradation of open circular and linear DNA, leaving ccc DNA largely intact. The remaining degradation was also

Figure 46

Non-specific intracellular DNases of *D.radiodurans* visualised in 5-15% SDS polyacrilamide gels containing native DNA (A) or heat denatured DNA (B).

Dark bands are labelled with capital letters and are regions of DNase activity. Individual lanes were developed in buffers containing different divalent cations. All lanes contained identical quantities of cell lysate from *D.radiodurans* wild type.

Lane	Buffer additions
1	no divalent cations
2	Ca^{2+}
3	Mg^{2+}
4	Mn^{2+}
5	Ca^{2+} and Mg^{2+}
6	Ca^{2+} and Mn^{2+}
7	Mg^{2+} and Mn^{2+}
8	Mg^{2+}, Mn^{2+} and Ca^{2+}


Gel lane numbers
1 2 3 4 5 6 7 8
A
intracellular DNA
A
B
C
J
L
M
Bands of DNA degradation
Gel lane numbers
1 2 3 4 5 6 7 8
B
B
C
D
E
F
G
H
I
J
K
L
M
Bands of DNA degradation

		Divalent cation requirement							
		Gel containing double stranded DNA				Gel containing single stranded DNA			
Band	*Approx. MW	no ions	Mg^{2+}	Ca^{2+}	Mn^{2+}	no ions	Mg^{2+}	Ca^{2+}	Mn^{2+}
A	130000	–	–	+	–	–	–	–	–
B	117000	–	–	+	–	–	–	+	–
C	105000	–	–	+	–	–	–	+	–
D	98000	–	–	±	–	–	–	+	–
E	90000	–	–	±	–	–	–	+	–
F	87000	–	–	±	–	–	–	+	–
G	71000	–	–	–	–	–	–	–	+
H	54000	–	–	–	–	–	–	–	+
I	48000	–	–	–	–	–	–	–	+
J	32000	+	+	+	+	–	–	–	+
K	22000	–	–	–	–	+	+	+	+
L	20000	+	+	+	+	+	+	+	+
M	19000	+	–	±	+	–	–	+	+

Figure 47

Summary of divalent cation requirements of the different bands of DNase activity observed in SDS polyacrilamide gels containing DNA.

- \- Indicates that the band of degradation does not appear.
- \+ Indicates that the band of degradation appears.

* calculated from Rf values relative to Rf values of molecular weight markers stained with coomassie blue.

Figure 48

Non-specific DNase activities in repair-deficient strains of *D.radiodurans* visualised in a 5-15% SDS polyacrilamide gel containing 20 $\mu g ml^{-1}$ heat denatured salmon sperm DNA.

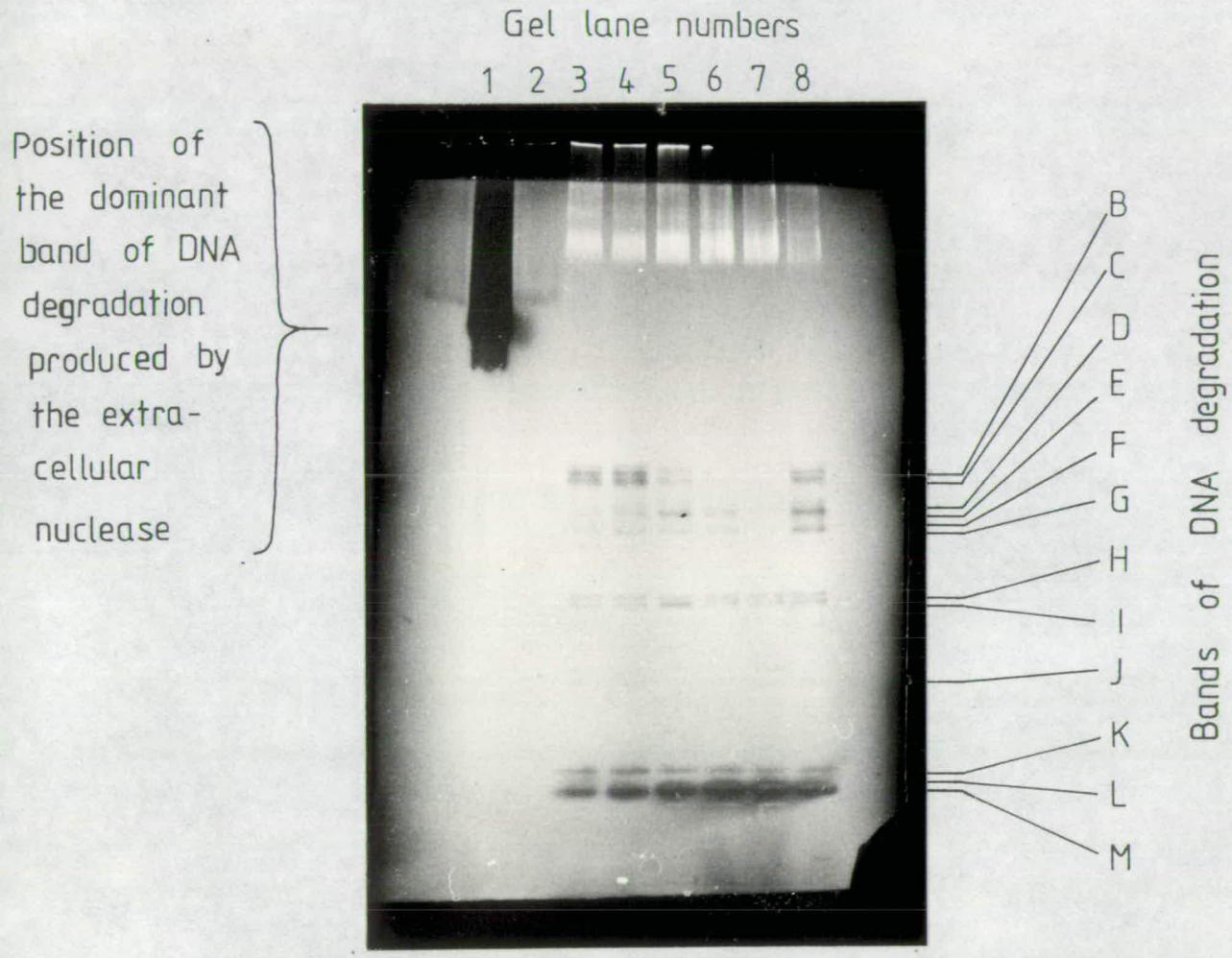
Lanes 1 and 2 were loaded with identical quantities of growth medium (cell free) obtained from stationary phase cultures of *D.radioduran* wild type and *nuc1* respectively.

Lanes 3 to 8 were loaded with cell lysates from repair-deficient strains and *D.radiodurans* wild type.

Lanes

1 wild type strain growth medium
2 *nuc1* strain growth medium
3 strain 251
4 strain 781
5 strain 25
6 strain 78
7 strain 302
8 wild type strain

The gel was developed in the presence of Mg^{2+}, Ca^{2+} and Mn^{2+} ions at 5mM.


Gel lane numbers
1 2 3 4 5 6 7 8
Position of the dominant band of DNA degradation produced by the extra-cellular nuclease
B
C
D
E
F
G
H
I
J
K
L
M
Bands of DNA degradation

reduced in the presence of 25mM NaCl and could be prevented by the addition of 1mM EDTA indicating that divalent cations were present in the cell lysate. It was hoped therefore that a balance had been struck between the requirement for suppression of the non-specific activities whilst retaining enough divalent cations should a repair enzyme require them. The residual non-specific DNase activity was more apparent at higher concentrations of cell-lysates and would destroy all substrate DNA. A series of two-fold dilutions of lysates of washed *D.radiodurans* in 25mM NaCl were therefore examined for specific activity towards UV irradiated ccc DNA so that at one dilution (which varied with different attempts), the extract was just dilute enough to prevent undamaged ccc DNA from being totally degraded whilst providing as concentrated a cell extract as possible. Specific degradation of a proportion of UV-irradiated ($90Jm^{-2}$) ccc DNA was observed at a narrow range of dilutions. Linear and oc DNA were both non-specifically degraded at these dilutions. Attempts were then made to enhance this activity by adding cofactors in various combinations which could favour the UV-specific activity and/or suppress the non-specific DNA degradation. EDTA (5mM) abolished both UV-specific and non-specific activities; mercaptoethanol (5mM) and ATP (5mM) had no effect. Increasing the NaCl concentration enhanced the UV-specific degradation which became apparent in a wider range of dilutions of cell lysate. The NaCl concentration was optimised at 100mM (Fig.49A). At this concentration bands were frequently observed after electrophoresis of the reaction products in agarose gels which were neither ccc DNA, open circular, or linear DNA and which may have been

Figure 49

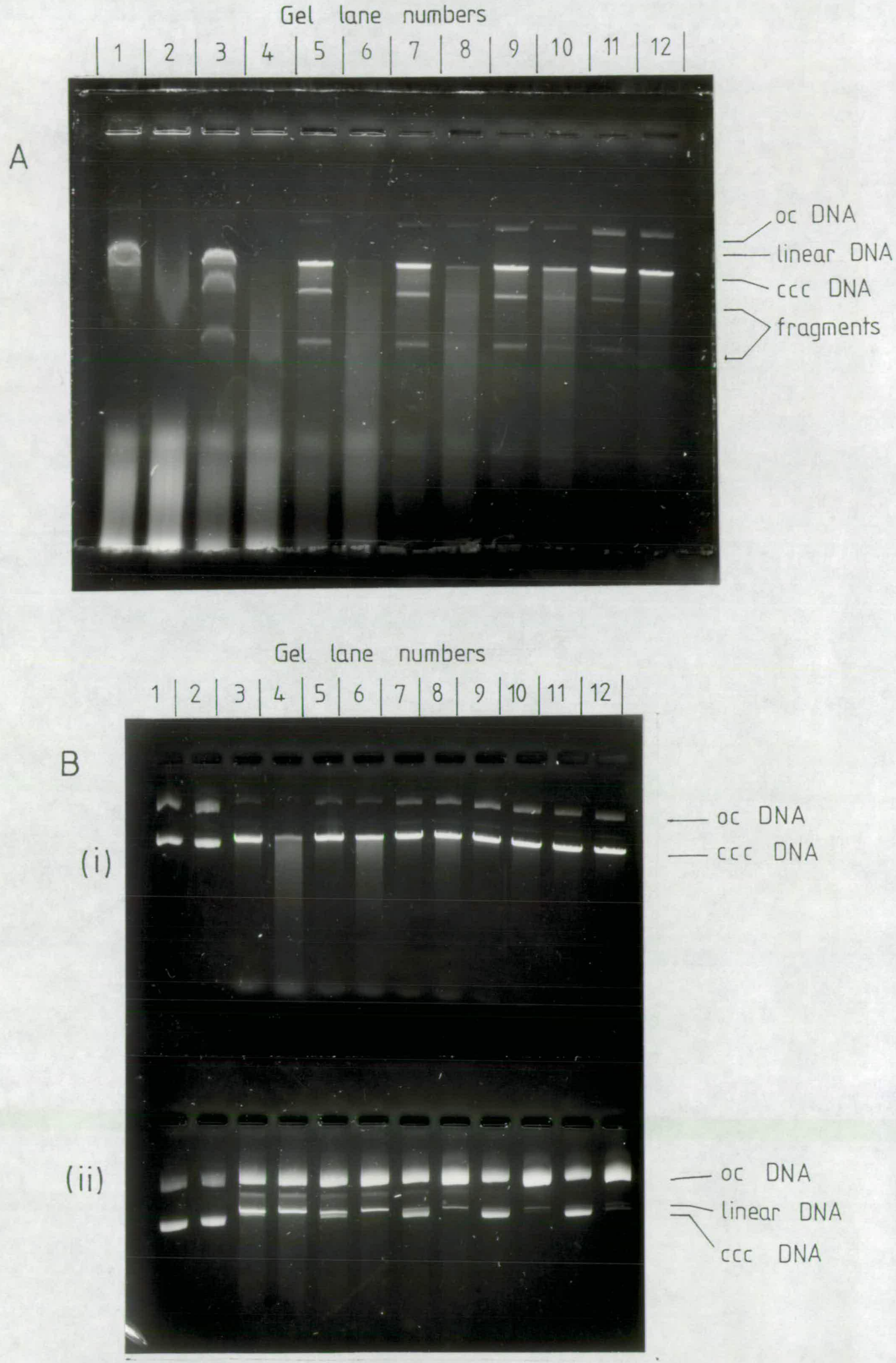
A. Typical pattern of UV-specific degradation of plasmid pML2 DNA obtained with different dilutions of cell free extracts from *D.radiodurans*.

Extract Dilution	Undamaged plasmid lanes	UV irradiated ($90Jm^{-2}$) plasmid lanes
none	1	2
x2	3	4
x4	5	6
x8	7	8
x16	9	10
x32	11	12

B. UV-specific conversion of covalently closed circular pML2 DNA to open circular form by the 0-45% and the 45-65% ammonium sulphate fractions obtained from *D.radiodurans*

(i) 0-45% (ii) 45-65%

Final protein concentration $mgml^{-1}$	Undamaged plasmid Lanes	UV irradiated ($90Jm^{-2}$) plasmid Lanes
none	1	2
2	3	4
1	5	6
0.5	7	8
0.25	9	10
0.125	11	12


Gel lane numbers
1
2
3
4
5
6
7
8
9
10
11
12
A
oc DNA
linear DNA
ccc DNA
fragments
Gel lane numbers
1
2
3
4
5
6
7
8
9
10
11
12
B
(i)
oc DNA
ccc DNA
(ii)
oc DNA
linear DNA
ccc DNA

restriction products of pML2 since *D.radiodurans* is known to possess a restriction enzyme Mra1 (Wani *et al.*, 1982). The reaction time was optimised at 30 min below which the UV-specific effect was less marked and above which non-specific activity began to mask the UV-specific effect.

A similar UV-specific degradation of ccc DNA was found in *D.proteolyticus* but not in *D.radiopugnans* or *D.radiophilus*. The UV-specific activity in *D.radiodurans* lysates survived at 0°C for several hours but was absent after 18 hours at 4°C. However, lysates could be frozen at -70°C for up to two weeks and would still display the same activity upon gentle thawing at 4°C.

Concentration of the UV specific activity

Attempts were made to concentrate the UV-specific endonuclease activity by ammonium sulphate precipitation which resulted in the optimised procedure described in materials and methods. The UV-endonuclease activity precipitated from crude extracts at approximately 55% $(NH_4)_2SO_4$ saturation; however the highest yields were obtained in 45-65% $(NH_4)_2SO_4$ cuts (Fraction A). The majority of non-specific nuclease activities precipitated between 0-40% $(NH_4)_2SO_4$ saturation and the residual non-specific activity in 'fraction A' was enhanced as the NaCl concentration was lowered. UV-endonuclease activity was still distinguishable if 'fraction A' was diluted to approx.20$\mu g\ ml^{-1}$ of total protein indicating that the activity had been significantly concentrated by ammonium sulphate precipitation (Fig.49B and Table 16). The activity prior to ammonium sulphate precipitation was typically 8000 units ml^{-1}

(approx.50mg ml^{-1}) and after precipitation as fraction A was approx.10000 units ml^{-1} (15mg ml^{-1}) (1 unit is defined as the minimum activity required to convert 1µg of UV-irradiated ccc DNA to oc DNA in 30 min at 30°C). Typically 4-6 ml of 'fraction A' could be obtained from a stationary-phase, 3 litre culture of wild type *D.radiodurans*.

Fraction A also contained chromosomal DNA (approx.40µg ml^{-1}) which was not sufficient to interfere with the assay of UV endonuclease activity on agarose gels. Attempts to remove cell DNA prior to ammonium sulphate precipitation by selective precipitation with 0.8% streptomycin sulphate eliminated the activity which was probably due to its being precipitated along with the DNA since the streptomycin sulphate itself was found not to destroy the activity. However, the precipitate was completely insoluble and no UV-endonuclease activity could be recovered from it.

During the attempts to determine at what point the UV endonuclease activity precipitated with ammonium sulphate another activity was noted which altered the mobility of ccc DNA in agarose gels whether DNA was irradiated or not (Fig.50). Plasmid pAT153 (supercoiled ccc form) migrated smaller distances after incubation with a 40-50% ammonium sulphate cut from the wild type for 30 min. The new position of the DNA band did not correspond with the positions of open circular or linear forms of the plasmid and may therefore have been due to partial relaxation of the supercoiling in the ccc form. The effect was less marked when pML2 was the substrate but resulted in the ccc pML2 band becoming tighter and more compressed as the protein concentration was decreased.

Figure 50

An activity from the 40-50% ammonium sulphate cut of *D.radiodurans* which alters the mobility of separated DNA without endonuclease action.

Final protein concentration ($\mu g ml^{-1}$)	pML2 lanes	pAT153 lanes
0	1	2
100	3	4
50	5	6
25	7	8
12	9	10
6	11	12

15 µg of extract in 10mM tris HCl pH 7.5 was incubated for 20 minutes at room temperature with 5 µl plasmid DNA (1 µg) before addition of STOP buffer.


Gel lane numbers
1
2
3
4
5
6
7
8
9
10
11
12
oc ML2
ccc ML2
oc pAT153
linear pAT153
ccc pAT153

Reaction conditions and properties of the UV-endonuclease activity

Fraction A was used to determine the optimal reaction conditions for the UV-endonuclease activity. The activity was maximal between 80mM to 150mM NaCl. NaCl could be replaced by KCl or Tris-HCl buffer at the same molarity. The pH range of the reaction was determined in 0.1M Tris buffer or 0.1M sodium acetate buffer at the appropriate pH to be 5.5 to 8.0 (Fig.51A). Non-specific nucleases were revealed at higher pH values and caused the production of linear DNA from the ccc and oc forms of pML2 DNA. The non-specific activity was suppressed below 7.0 and so subsequent assays and purification was carried out at pH 7.0 or below. The UV-specific activity was inactivated by 0.1mM EDTA. If the activity was exposed to 2mM EDTA for 5 min and then excess (5mM) divalent cations added the activity was not restored. The activity was also eliminated if the EDTA was saturated with excess $CaCl_2$ before addition to fraction A. This meant that EDTA had to be excluded from all buffers in the preparation of fraction A and the buffers used in the final steps of plasmid preparations. This was not done in early attempts to identify UV-specific activity and probably explains the failure to detect the UV-endonuclease activity. The dialysis membranes used were originally prepared by boiling in the presence of EDTA but the EDTA was found to persist even after extensive washing and inactivated the UV-endonuclease activity. This sensitivity to EDTA indicated that the UV endonuclease activity was divalent-cation requiring and so an attempt was made to determine which ion was required. Enhancement of activity was measured

Figure 51

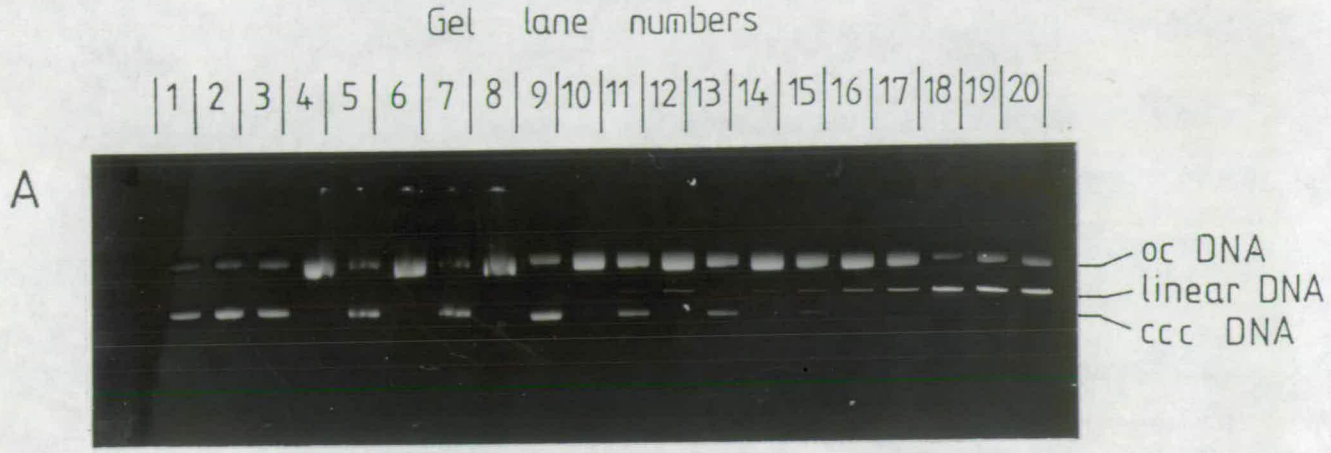
A. The effect of pH on incision of UV-irradiated plasmid DNA by the 45-65% ammonium sulphate fraction from *D.radiodurans* wild type.

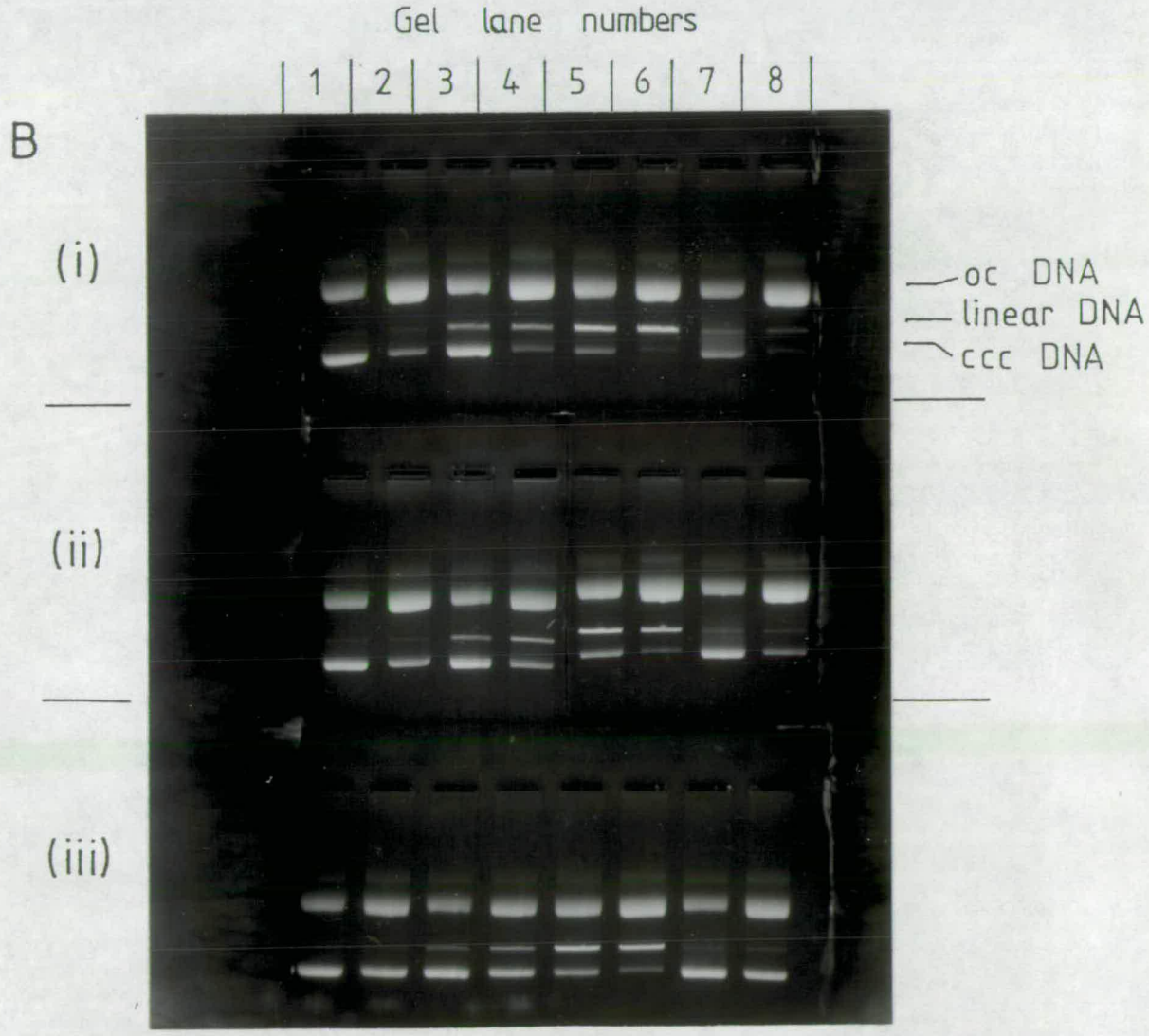
pH	Undamaged plasmid lanes	UV irradiated ($90Jm^{-2}$) plasmid lanes
5.0	1	2
5.5	3	4
6.0	5	6
6.5	7	8
7.0	9	10
7.5	11	12
8.0	13	14
8.5	15	16
9.0	17	18
9.5	19	20

B. The effect of divalent cations on incision of UV-irradiated plasmid DNA by the 45-65% ammonium sulphate fraction from *D.radiodurans* wild type.

Protein conc. (i) $20\mu gml^{-1}$ (ii) $10\mu gml^{-1}$
(iii) $5\mu gml^{-1}$

Divalent cations	Undamaged plasmid lanes	UV-irradiated ($90Jm^{-2}$) plasmic lanes
none	1	2
Mg^{2+}	3	4
Mn^{2+}	5	6
Ca^{2+}	7	8


Gel lane numbers
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
A
oc DNA
linear DNA
ccc DNA


Gel lane numbers
1 2 3 4 5 6 7 8
B
(i)
(ii)
(iii)
oc DNA
linear DNA
ccc DNA

empirically by either diluting the enzyme such that in the time normally allowed for the reaction approximately half the ccc DNA was incised (Fig.51B), or alternatively, an excess of enzyme was used and the time allowed for incision shortened so that about half the DNA remained unincised (approximately 15min incubation). In both cases it was assumed that increased activity would result in the complete degradation of the remaining unincised fraction. In both methods 5mM Mg^{2+}, Ca^{2+} or Mn^{2+} failed to enhance the activity whereas 5mM Zn^{2+} was inhibitory. The former three increased non-specific incision of the substrate whereas Zn^{2+} did not. The UV-endonuclease activity also appeared to act independently of an external energy source and 5mM ATP failed to enhance the activity. A reducing environment produced by 40mM mercapto-ethanol or dithiothreitol also did not influence the activity. 50mM caffeine added to the reaction mixture failed to inhibit the activity. However, acriflavin prevented all activity at $10\mu gml^{-1}$ but had no effect at $1\mu gml^{-1}$. 25mM caffeine and $1\mu gml^{-1}$ of acriflavin have both been shown to inhibit UV-specific incision in *E.coli* (Seeberg and Strike 1976). The activity was stable for 30 min at 50°C but was rapidly (under 1 min) inactivated at 55°C. PM2 DNA was not used in the assay of this enzyme since it was found that a contaminating substance (still present after CsCl centrifugation) inhibited the UV endonuclease activity. The substance did not interfere with the action of the HUV endonuclease activity (see next section) or non-specific nucleases. pML2 did not suffer from this problem and was therefore routinely used as substrate for this UV endonuclease activity. These characteristics are summarised in Table 15.

Measurement of pyrimidine dimer glycosylase activity

If the UV endonuclease was similar to the T4 or *M.luteus* pyrimidine dimer glycosylases it would cleave the N-glycosyl bond of the 5' pyrimidine in cyclobutane dimers, the 5' pyrimidine remaining attached to the 3' pyrimidine of the dimer (and hence DNA) by the cyclobutane ring. Subsequent irradiation (photoreversal) of the dimer with 254nm light would liberate the 5' pyrimidine from the DNA. Such liberation would not occur if incision proceeds *via* true endonuclease action such as *via* the UVRABC enzyme (Friedberg *et al.*, 1981). The liberated 5' pyrimidine is soluble in 0.067M sodium carbonate/0.067M zinc acetate whereas nucleotides, oligonucleotides and DNA are not (Bonura *et al.*, 1982). *D.radiodurans* ^{3}H DNA was therefore UV irradiated with 2000 Jm^{-2} UV to convert 3-4% of ^{3}H labelled thymines to dimers. DNA (50000 cpm, 5µg) was incubated with 500 units of UV endonuclease or 500 units of *M.luteus* UV glycosylase under optimal conditions for 1 hr. The mixtures (100 µl) were then irradiated with >12000 Jm^{-2} 254nm UV light (15 min 1.5 cm from the UV tube) which should photoreverse >70% of liberatable thymine (Friedberg *et al.*, 1981). DNA, oligonucleotides, nucleotides and protein were precipitated by adding equal volumes of 0.2M sodium carbonate and 0.2M zinc acetate. After centrifugation the radioactivity in the supernatant was determined in dioxan based scintillant (NE255). The *D.radiodurans* UV endonuclease did not generate free thymine (see below).

	Radioactivity in supernatant (cpm)
(a) DNA + 2000 Jm^{-2}UV	40
(b) DNA + 2000 Jm^{-2}UV + >12000 Jm^{-2}UV	32
(c) as (a) + *M.luteus* UV glycosylase	52
(d) as (b) + *M.luteus* UV glycosylase	1658
(e) as (a) + *D.radiodurans* UV endonuclease	43
(f) as (b) + *D.radiodurans* UV endonuclease	41

DNA repair-deficient strains lacking the UV endonuclease activity

Nineteen DNA repair deficient strains were examined for their ability to degrade UV damaged DNA. The UV endonuclease activity was determined in crude lysates at a variety of dilutions of lysate and in most cases the fraction A was obtained and also assayed. All the MMS-resistant strains described earlier except strain 302 and the wild type strain lacked the UV specific activity (Fig.52A and 52B, Table 14) whereas all the MMS-sensitive strains and the rec30 strain possessed the activity. The results clearly identify three mutations present in strains 781, 251 and 91 as eliminating the activity.

A second UV endonuclease activity

The six strains that lacked the UV endonuclease all showed a residual UV-specific nicking when 'fraction A' was assayed. This was manifested as a UV-specific incision of a small fraction of UV-irradiated plasmid molecules which did not increase with increasing concentrations of protein, indicating that not all the UV damage was being acted upon. This may have been due to leaky mutations or to the presence of another enzyme that was recognising a minor form of UV damage in the plasmid DNA. The latter was found to be the case since increasing the quantity of UV damage increased the proportion of incised molecules (Fig.52B). This would not be expected if the residual activity was due to a partially disabled UV endonuclease. All molecules were incised by the second activity when the substrate was irradiated with about $600J,^{-2}$ UV, whereas the predominant

Figure 52

A. Degradation of UV-irradiated plasmid DNA by 4 fold diluted cell free extracts of DNA repair deficient strains of *D.radiodurans*.

Strain	Unirradiated plasmid lanes	UV irradiated ($90Jm^{-2}$) plasmid lanes
wt	1	2
302	3	4
78	5	6
781	7	8
131	9	10
1311	11	12

B. Conversion of UV-irradiated ccc plasmid DNA to open circular form by the 45-65% saturated ammonium sulphate extracts obtained from repair deficient strains.

(i) Wild type strain (ii) strain 78 (iii) strain 781

All lanes contain $100\mu gml^{-1}$ of protein extract. The plasmid DNA was irradiated with increasing doses of UV from left to right.

Lanes		Lanes	
1	$0Jm^{-2}$	7	$150Jm^{-2}$
2	$5Jm^{-2}$	8	$200Jm^{-2}$
3	$10Jm^{-2}$	9	$300Jm^{-2}$
4	$20Jm^{-2}$	10	$400Jm^{-2}$
5	$40Jm^{-2}$	11	$500Jm^{-2}$
6	$60Jm^{-2}$	12	$600Jm^{-2}$

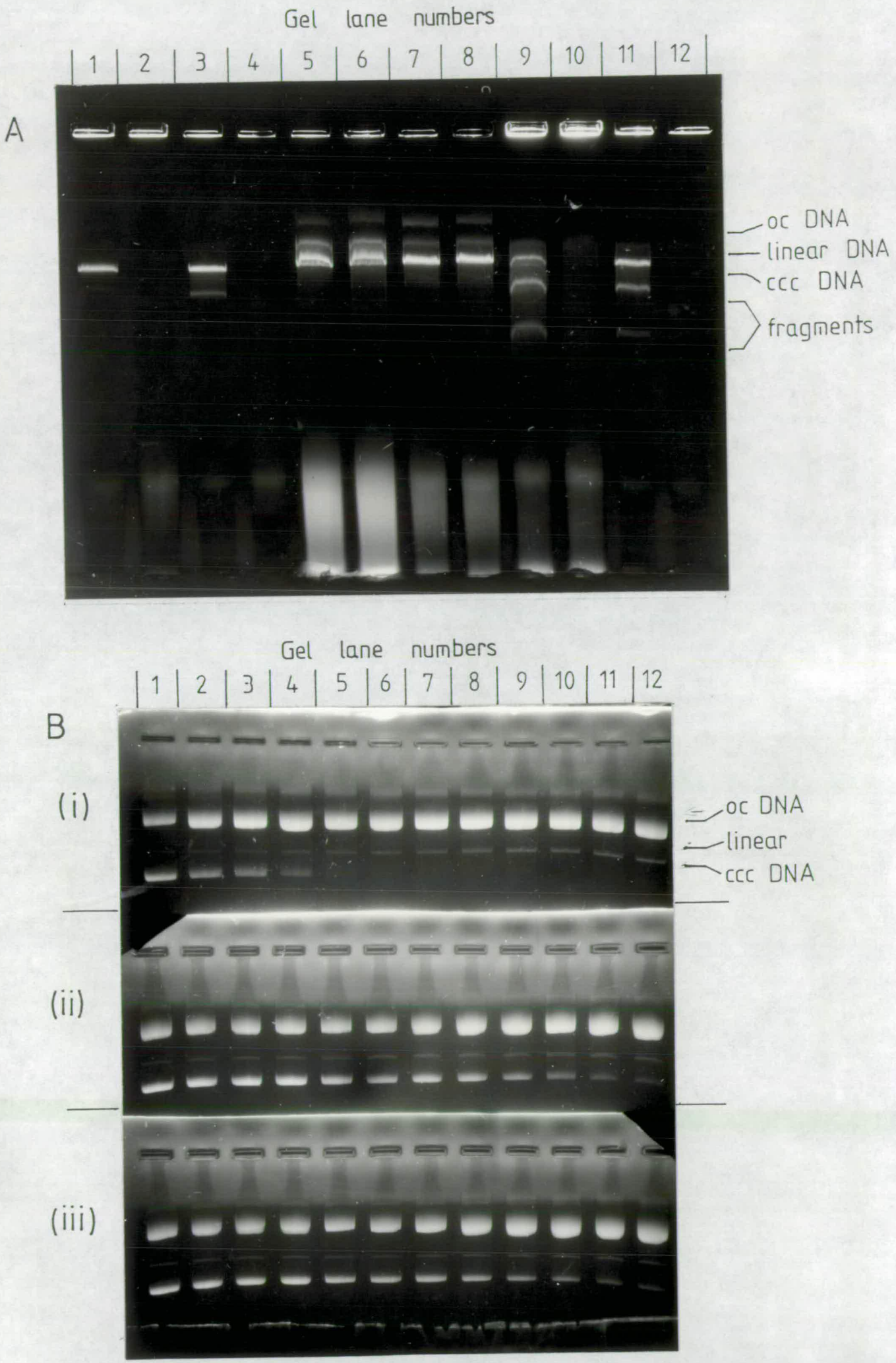

Gel lane numbers
1
2
3
4
5
6
7
8
9
10
11
12
A
oc DNA
linear DNA
ccc DNA
fragments
Gel lane numbers
1
2
3
4
5
6
7
8
9
10
11
12
B
(i)
(ii)
(iii)
oc DNA
linear
ccc DNA

Table 14

Summary of the UV specific incision of plasmid DNA by cell free extracts obtained from DNA repair-deficient strains of *D.radiodurans*.

* 45-65% ammonium sulphate cut

ND not determined

\+ UV specific incision of DNA

\- no UV specific incision of DNA

Strain	Crude lysate	AS* extracts	Strain	Crude lysate	AS* extract
Wild type	+	+	131	+	+
302	+	+	1311	+	ND
9	—	—	128	+	+
91	—	—	1281	+	ND
25	—	—	112	+	+
251	—	—	8	+	+
78	—	—	301	+	+
781	—	—	303	+	+
rec 30	+	ND	261	+	ND

Table 15 Summary of the chacteristics of the DNA endonuclease activities observed in *D. radiodurans*.

	Low UV endonuclease	High UV endonuclease[1]	AP endonuclease	Extracellular endonuclease	Non-specific intracellular endonuclease
pH	5·5 –> 8·5	5·5 –> 8·5	5·5 –> 8·5	5·0 — 9·0	7·5 –> 9·0
Ionic conc.	80 – 150 mM	0 – 200 mM	0 – 200 mM	0 – 500 mM	0 – 50 mM
Ammonium sulphate cut	50 – 60% sat.	45 – 65 % sat.	45 – 65% sat.	15 – 45% sat.	15 – 45% sat.
EDTA	Inhibited by 0·1 mM	Active at 10 mM	Active at 10 mM	Reversibly inhibited	Inhibited by 10 mM
Divalent ions	Mn^{2+} reqd. Cu^{2+}, Zn^{2+} inhibits	No effect	No effect	Stimulated by $Mn^{2+} > Mg^{2+} > Ca^{2+}$	Stimulated by Mn^{2+}
5mM ATP	No effect	No effect	No effect	ND	ND
50mM caffeine	No effect	No effect	No effect	ND	ND
Acriflavin 10 $mgml^{-1}$	Inhibits	No effect	ND	ND	ND
NEM	10 mM Inhibits	Active at 20 mM	ND	ND	ND
Mercapto ETOH or Dithiothrietol	No effect at 10 mM	No effect at 10 mM	No effect at 10 mM	No effect at 10 mM	Inhibited by 5 mM
Minimum time to digest 1 µg DNA[2]	25 min.	25 min.	10 min.	3 min.	5 min.
Stability on dilution	Labile	Stable	Stable	Stable	Stable
Survival at 4°C	5 days if conc. above 1 $mgml^{-1}$	5 days	5 days	> 2 weeks	ND
Survival at -70°C	> 5 months	> 5 months	> 5 months	> 1 month	> 3 months

NEM = N-ethylmaleimide ETOH = Ethanol 2 at 30° C under optimal conditions

1 also endonuclease activity towards OsO_4 treated DNA

activity in the wild type extracts completely incised plasmid DNA irradiated with $30Jm^{-2}$ UV. The latter activity is therefore referred to as the "low UV (LUV) endonuclease" and the former as the "high UV (HUV) endonuclease". Examination of the reaction properties of this latter activity, which was carried out as with the LUV endonuclease activity (see Table 15), showed it to differ most markedly in its resistance to 10mM EDTA and 20mM N-ethylmaleimide (NEM). This resistance allowed demonstration of the HUV endonuclease in wild type extracts since the LUV endonuclease is suppressed by EDTA. Similarly the LUV endonuclease activity could be suppressed in the wild type by using PM2 DNA as substrate or by the addition of N-ethylmaleimide. The minimum UV dose which allowed complete incision of 1µg of ccc DNA by the HUV endonuclease was about 20 fold higher (about $600Jm^{-2}$) than that required by the LUV endonuclease (about $30Jm^{-2}$). As with the LUV endonuclease the complete incision of UV-irradiated DNA (1µg) took longer than expected *in vitro* taking 35 to 40 min. This was constant over a 100 fold range of protein concentrations and could be reduced to 25 to 30 min if the ionic concentration was increased to 100mM. The unit of HUV endonuclease activity is therefore defined as for the LUV endonuclease. The HUV endonuclease was not observed clearly in crude extracts but 5000-6000 units ml^{-1} were present in fraction A from the wild type strain and also strains UVS9, UVS25, UVS78, 302, 131, 128, 112 and 8.

One possible lesion that could serve as substrate for the HUV endonuclease is the Thy (6,4) adduct. This is converted back to the original pyrimidines by irradiation at 303mM (Jagger 1976). Irradiation of PML2 DNA containing approximately

1 HUV endonuclease substrate per plasmid molecule with approximately $10^4 Jm^{-2}$ of 300nm light (by placing the DNA on a UV transilluminator (302 nm) for 30 min in a petri dish (to exclude <300 nm light)) failed to remove the HUV substrate indicating that Thy (6,4) pyo adducts are not the lesion recognised by the HUV endonuclease.

The reaction characteristics in the presence of different concentrations of salt, mercaptoethanol, diothiothreitol, caffeine and acriflavin, and the pH range of the activity were determined and are summarised in Table 15.

Other DNA damage-specific endonuclease activities

The presence of the two activities towards UV-irradiated DNA within fraction A in the wild-type strain prompted a search for activity toward depurinated DNA, since AP endonuclease activity is associated with UV glycosylases (see introduction). Activity against heat-depurinated DNA was found in the wild type in as large amounts as the two UV endonuclease activities. The AP endonuclease activity was fast acting in comparison with the UV endonuclease activities, 1µg of depurinated DNA being incised by an excess of AP endonuclease activity in 10 min. Investigation of its preferred reaction environment (Table 15) revealed a broad pH optimum and absence of a requirement for divalent cations or other cofactors. This activity was present in similar quantities in strains 9, 25, 78, 91, 251, 781, 131, 303, 128, 112 and 8 (Fig.53A).

Other damage-specific activities detected in fraction A from the wild-type strain were observed in DNA damaged with MMS (methylated DNA), EMS (ethylated DNA) and 8 methoxypsoralen

Figure 53

A. The effect of the 45-65% ammonium sulphate extracts from DNA repair-deficient strains on depurinated plasmid DNA.

(i) strain 78 (ii) strain 25 (iii) strain 9

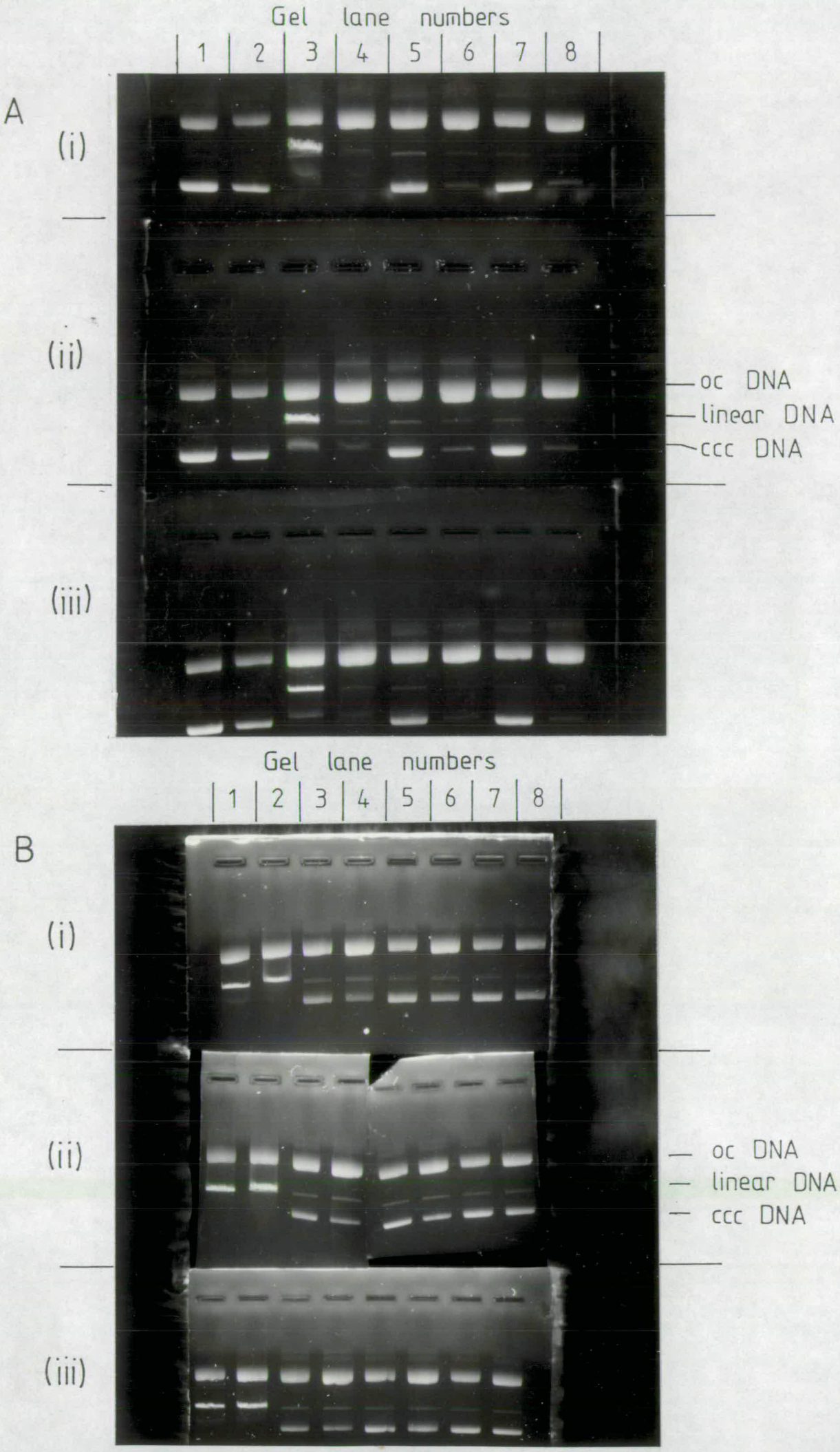
Concentration of protein	Undamaged plasmid lanes	Depurinated plasmid lanes
none	1	2
$2mgml^{-1}$	3	4
$1mgml^{-1}$	5	6
$0.5mgml^{-1}$	7	8

Similar results were obtained with MMS-treated DNA as substrate.

B. The effect of recombining 45-65% ammonium sulphate fractions from strains lacking UV endonuclease activity towards lightly irradiated DNA.

(i) strains 78 and 25 mixed 1:1
(ii) strains 78 and 9 mixed 1:1
(iii) strains 25 and 9 mixed 1:1

Final protein conc.	Undamaged plasmid	UV irradiated ($90Jm^{-2}$) plasmid
$2mgml^{-1}$	1	2
$1mgml^{-1}$	3	4
$0.5mgml^{-1}$	5	6
$250\mu gml^{-1}$	7	8


Gel lane numbers
1
2
3
4
5
6
7
8
A
(i)
(ii)
(iii)
oc DNA
linear DNA
ccc DNA
Gel lane numbers
1
2
3
4
5
6
7
8
B
(i)
(ii)
(iii)
oc DNA
linear DNA
ccc DNA

+ near UV light (DNA crosslinks). The last activity was weak and resulted in only a proportion of the ccc DNA being converted to oc DNA. An activity towards OsO_4 treated DNA (pyrimidine hydrates) was found in Fraction A in similar amounts to the HUV endonuclease activity. It also had an identical pH range and resistance to N-ethylmaleimide, EDTA, heat and NaCl as the HUV endonuclease. It was also present in strains UVS78, 781 and 302.

Attempts to reconstitute the LUV endonuclease activity

The LUV endonuclease activity was eliminated in strains 9, 25, 78, 91, 251 and 781 by three independent mutations suggesting that the activity was the product of more than one gene. Crude lysates or the fraction A from strains 9, 25 and 78 or strains 91, 251 and 781 were mixed in equal proportions in all permutations in an attempt to allow the deficiency induced by one mutation to be complemented by the extract from a different strain. However, LUV endonuclease activity failed to reappear in any combination after several attempts (Fig.53B).

Polyacrylamide gel electrophoresis of proteins

The proteins in crude lysates and the fraction A extracts from the wild type and DNA-repair deficient strains were separated in SDS containing gradient polyacrylamide gels and visualised by staining with kenacid blue or silver in an attempt to identify differences that could be attributed to the presence of particular mutations. No differences consistent with the presence or absence of particular mutations were observed in

the proteins from crude lysates (Fig.54A). In contrast proteins separated from the fraction A extracts displayed several differences between the DNA repair-deficient strains and the wild type, particularly in extracts from strains 25 and 251 where the higher molecular weight proteins in general were absent (Fig.54B). These differences persisted when the fraction A extracts were carefully reprepared on several occasions and were not altered if the fraction A extracts were prepared in the presence of 10mM EDTA and 0.1mM PMSF in all buffers to suppress protease activity. The absence of high molecular weight proteins in strains 25 and 251 cannot be explained but appears to be a function of mutations common to both strains. No single protein could be definitely identified as being specific to a particular mutation in other strains. A protein band of approx. MW 50000 was absent from strains 302 and 78 but present in strain 781 and the wild type which would be consistent with the presence of a functional *mtcA* gene. The protein appears to be present in strain 9 however which lacks a functional *mtcA* gene.

Further purification of the UV endonuclease activities

Attempts were made to separate the UV endonucleases from each other and other non-specific nucleases by ion exchange chromatography. The LUV and HUV endonucleases in Fraction A were found to bind to DEAE sephacel and to hydroxylapatite but not to CM cellulose between pH 6.0-9.0 in test-tube experiments and were subsequently released at pH 7.0 by increasing the ionic strength of the buffers to 0.5M NaCl for DEAE sephacel and 0.4M phosphate for hydroxylapatite. Fraction A (50mg of protein) was therefore

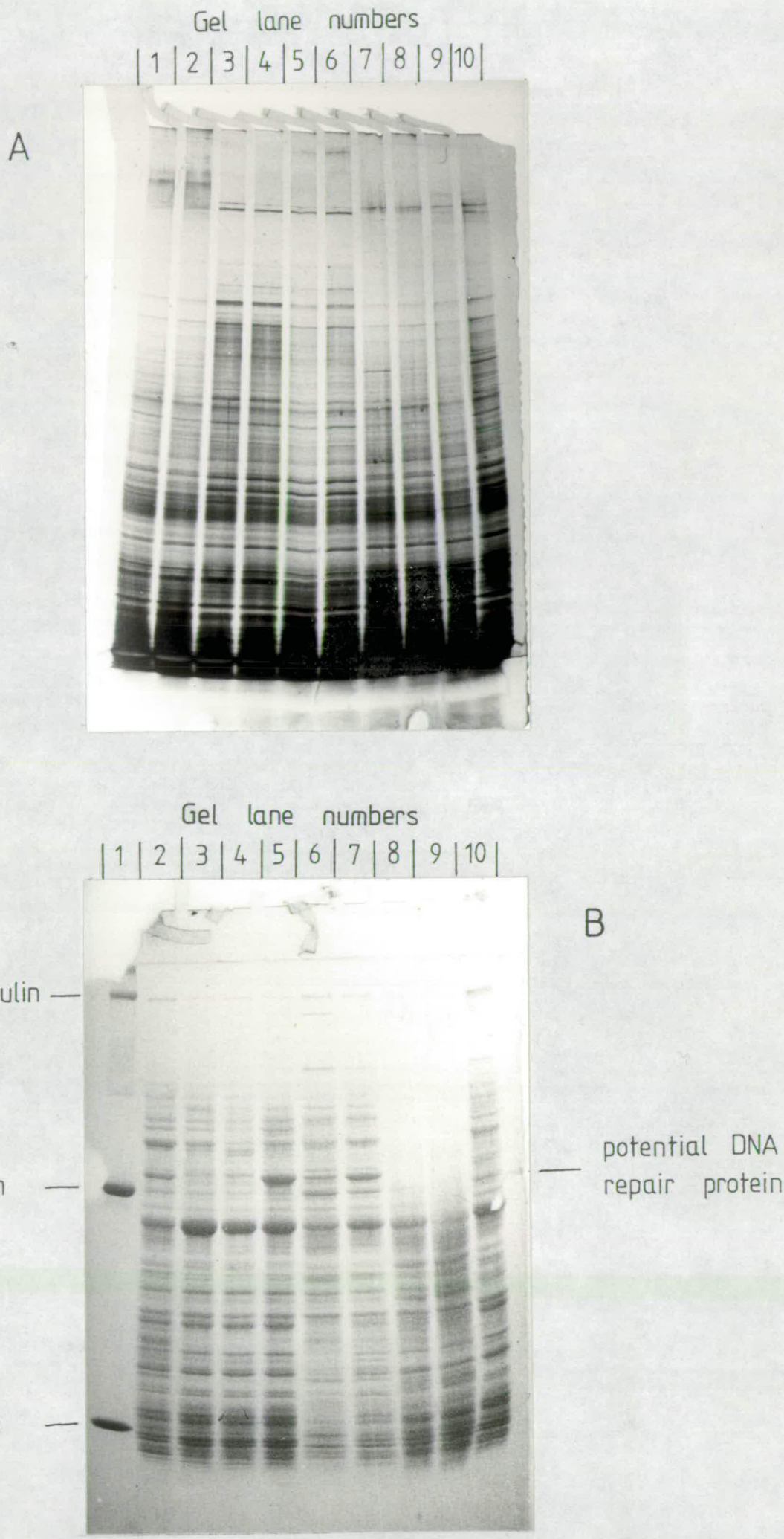
Figure 54

A. SDS polyacrylamide gel (7-15%) of total proteins from lysates of repair deficient strains of *D.radiodurans*.

Lanes		Lanes	
1	strain 303	6	strain 25
2	strain 301	7	strain 781
3	strain 91	8	strain 78
4	strain 9	9	strain 302
5	strain 251	10	wild type strain

B. SDS polyacrylamide gel (7-15%) of 45-65% ammonium sulphate fractions obtained from repair deficient strains of *D.radiodurans*.

Lanes		Lanes	
1	MW markers - lysozyme ovalbumin thyroglobulin	6	Strain 9
2	wild type strain	7	strain 91
3	strain 302	8	strain 25
4	strain 78	9	strain 251
5	strain 781	10	strain 8


Gel lane numbers
1 2 3 4 5 6 7 8 9 10
A
Gel lane numbers
1 2 3 4 5 6 7 8 9 10
B
thyroglobulin
ovalbumin
lysozyme
potential DNA repair protein

loaded onto a column of DEAE sephacel (2.6x10cm) previously equilibrated with 20mM Tris HCl pH 7.0. The column was washed with 200 ml of 20mM Tris HCl pH 7.0 at 40ml hr^{-1} then developed overnight (18 hr) with a linear gradient of 0→0.5M NaCl in 20mM Tris HCl pH 7.0. Non-specific nuclease activity appeared both in the wash and just before (∿0.20→0.30M NaCl) a single peak of LUV endonuclease activity eluting between 0.30-0.35M NaCl (Fig.55, Table 16). HUV endonuclease was assayed by including 1mM EDTA in assay mixtures which suppressed both the non-specific activities and LUV endonuclease activity. HUV endonuclease activity eluted at approx.0.23-0.30M NaCl (Fig.55, Table 17). LUV endonuclease was unstable at this point and rapidly decayed unless bovine serum albumin (BSA, 1mg ml^{-1}) was added to the collected fractions, in which case the activity was stable for 2 days at 4°C. The HUV endonuclease and the non-specific activity were stable for several days without added BSA. Fractions containing LUV endonuclease (+BSA) were combined, dialysed overnight against 4 litres of 10mM phosphate buffer pH 7.0, then loaded onto a column of hydroxylapatite (2.6x10 cm). The column was washed with 200ml of 10mM phosphate buffer (40ml hr^{-1}), then developed with a linear gradient of 0→0.4M phosphate buffer overnight (18 hr). Fractions were collected in the presence of BSA (1mg ml^{-1}). However, no fractions contained LUV endonuclease activity. The LUV endonuclease was therefore too unstable to survive passage through the hydroxylapatite column. A peak of non-specific activity appeared as a single peak at approx.0.15→0.20M phosphate. Fractions obtained from the DEAE sephacel column that contained HUV endonuclease activity were also combined, dialysed against 4 litres of 10mM phosphate

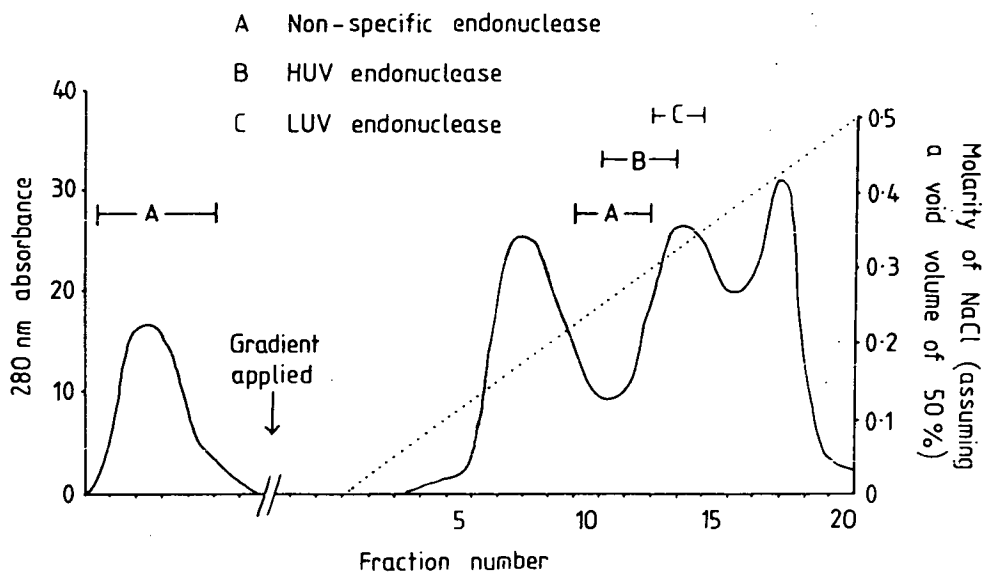


Figure 55

Elution of endonuclease activities in Fraction A from DEAE sephacel with increasing concentrations of NaCl.

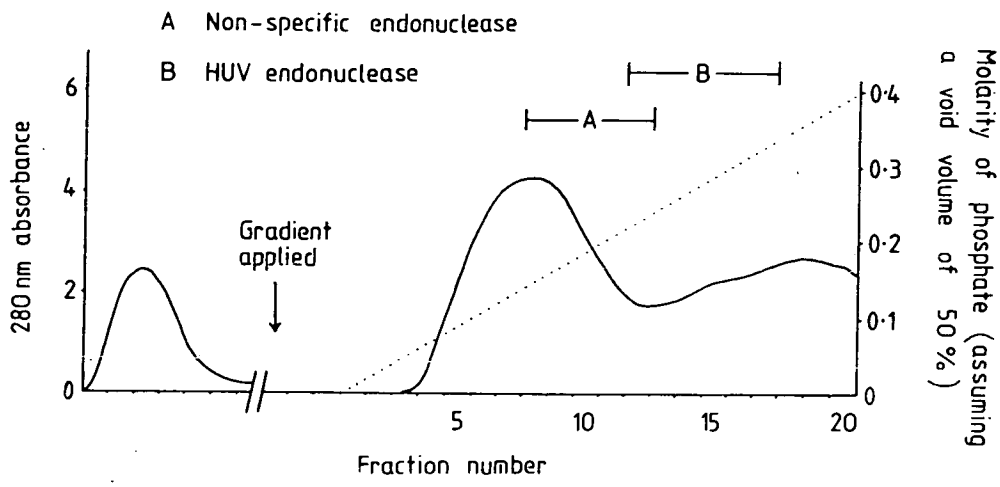


Figure 56

Elution of endonuclease activities from hydroxylapatite with increasing concentrations of phosphate.

Table 16 Purification of LUV endonuclease activity

Purification step	*Protein mg.*	*Units of activity*	*Specific activity units/mg*	*Yield %*
Crude extract	750	32000	43	100
45-65% $(NH_4)_2SO_4$	75	32000	427	100
50mg applied to DEAE sephacel	15	7400	490	23
150mg applied to DEAE sephacel 0.2M-0.4M step	70	64000	914	100
65% $(NH_4)_2SO_4$	20	25600	1280	40
ACA54	5	3000 ($-Mn^{2+}$)	600	0.4
		24000 ($+Mn^{2+}$)	4800	3.4

Table 17 Purification of HUV endonuclease activity

Purification step	*Protein mg.*	*Units of activity*	*Specific activity units/mg*	*Yield %*
Crude extract	750	?	?	?
45-65% (NH_4SO_4)	75	30000	400	100%
50mg applied to DEAE sephacel	10	6000	600	30%
Hydroxylapatite	2	4600	2300	23%
150mg applied to DEAE sephacel 0.2M-0.4M step	70	64000	914	106%
65% $(NH_4)_2SO_4$	20	25600	1280	42%
ACA54	6	20000	3330	33%

buffer pH 7.0 and applied to and eluted from a hydroxylapatite column (2.6x10cm) in a similar to the LUV endonuclease containing fractions. The HUV endonuclease eluted between 0.25→0.35M phosphate (Fig.56, Table 17). The activity had been separated from the non-specific activity and was apparently free of AP endonuclease activity.

Estimation of the molecular weight of damage specific endonucleases

Initial attempts to resolve proteins from wild-type Fraction A on a variety of gel filtration columns using low ionic strength buffers typically resulted in all proteins eluting as a single peak of molecular weight greater than 10^6 ie the proteins were binding together into a supercomplex. It was likely that this was due to a combination of factors such as a predominance of a highly charged protein, residual nucleic acid and low ionic environments. To overcome this problem Fraction A was first passed through a DEAE sephacel column as described earlier, except that because it was desirable to recover more than one activity, and to obtain maximum LUV endonuclease activity (because it denatures if left too long when dilute), 150 mg of Fraction A was applied to the column and eluted with a step gradient. The first step was 0.2M NaCl in 10mM Tris HCl pH 7.0 (30 ml, 15 ml hr^{-1}) and the second 0.4M NaCl in 10mM Tris HCl pH 7.0 (30 ml, 15 ml hr^{-1}). All the relevant activities were recovered in the second step. Two volumes of saturated $(NH_4)_2SO_4$ were added to this and the precipitate collected by centrifugation. The pellet was resuspended in 2 ml of 10mM Tris HCl buffer pH 7.0 which left about half the proteins as an insoluble precipitate. This was removed by centrifugation and the remaining soluble protein

supernatant filtered through a 0.45μm millipore filter, then dialysed overnight at 4°C against 10mM Tris HCl pH 7.0 0.2M NaCl. This extract contained both UV endonuclease activities and the endonuclease activity towards OsO_4 treated DNA. 0.5 ml quantities were loaded onto gel filtration columns equilibrated with 10mM Tris HCl, pH 7.0, 0.2M NaCl. Each column separated proteins of different molecular size. The best separation of the desired activities was achieved on a 1.5x70cm ACA54 column (L.K.B.) which separates molecules with molecular sizes (Stokes radii) in the region of 5000-70000 daltons (Fig.57). The column was calibrated by eluting proteins of known molecular weight. The volume of buffer required to elute each protein (elution volume) is linearly related to $\log_{10}$ molecular weight of the proteins, allowing the calibration shown in Fig.57. Molecular weight standards used were Blue dextran 2000 (Pharmacia, MW>70000), bovine serum albumin (Sigma, MW 68000), hen egg ovalbumin (Sigma, MW 45000), chymotrypsinogen A (Sigma, MW 25000), bovine ribonuclease A (Sigma, MW 14000) and potassium ferrocyanide (BDH, MW 367). The position of proteins was monitored by absorbance at 280mm which revealed sharp even peaks which typically were eluted in twelve ml volumes. Thirty-six fractions were collected and assayed for LUV endonuclease activity. HUV endonuclease and OsO_4 endonuclease activity were assayed after LUV endonuclease activity had been inactivated by the addition of EDTA (to 10mM) to fractions. The molecular size of LUV endonuclease was calculated to be approximately 36000 daltons by this method. The molecular size of the two remaining endonuclease activities was the same at approximately 27000

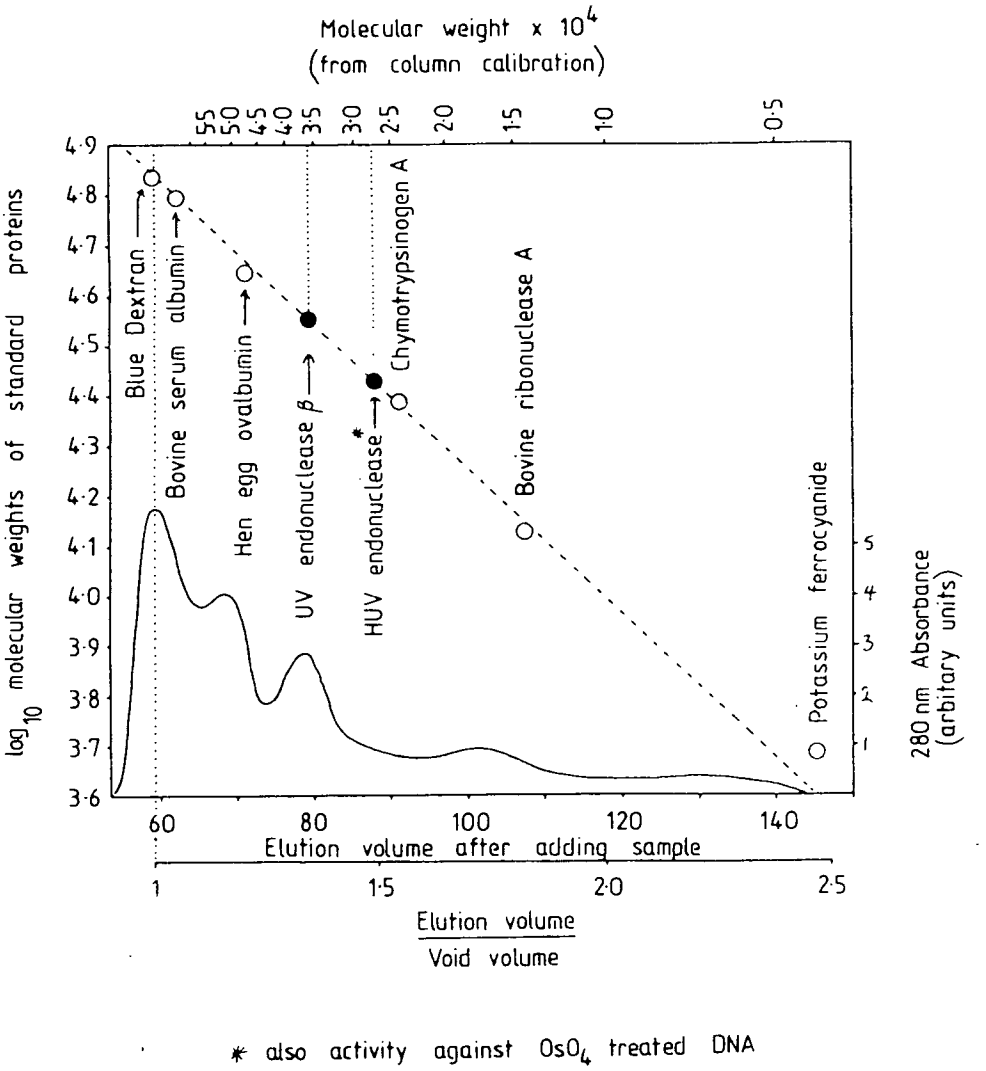


Figure 57

Position of elution of maximum LUV endonuclease activity (UV endonuclease β), HUV endonuclease activity and endonuclease activity towards OsO_4 treated DNA from an Ultragel ACA 54 gel filtration column.

daltons (Fig.57). The purification of the UV endonucleases is summarized in tables 16 and 17.

A non-specific activity which converted ccc DNA, apparently directly to linear forms was completely separated from the UV specific activities by passage through the ACA34 column. This enzyme had a molecular weight of greater than 50000 and was stimulated by Mn^{2+} ions (1mM $MnCl_2$).

The effect of divalent cations on LUV endonuclease activity eluted from the ACA34 column

The separation of the non-specific activity towards ccc DNA prompted examination of the effect of the divalent cations that promote non-specific activity in cruder preparations on the LUV endonuclease activity to assess its purity (since generally non-specific activities are non-requiring). No ion (Ca^{2+}, Mg^{2+} or Mn^{2+}) promoted non-specific cleavage of DNA in the LUV endonuclease or HUV endonuclease-containing fractions indicating that the UV endonucleases are free of non-specific endonucleaes. The enzymes were also free of non-specific exonucleases since linear DNA and open circular DNA were not degraded in the presence of any ion. Unexpectedly, Mn^{2+} ions increased the number of LUV endonuclease units of activity eight fold in these experiments in contrast to the lack of effect of ions in cruder preparations of enzyme. This enhancement was Mn^{2+} specific, Ca^{2+} or Mg^{2+} having no effect. Mn^{2+} ions also had the ability to reintroduce LUV endonuclease activity into enzyme samples that had lost activity in the absence of added ions by overnight incubation at 4°C. Mn^{2+} concentrations as low as 0.01mM produced the enhancement.

The reason for the enhancement appearing at this point is possibly that an activity which inhibits or degrades LUV endonuclease enzyme is also Mn^{2+} stimulated and this has been removed by gel filtration allowing the Mn^{2+} stimulation of UV endonuclease activity to become apparent.

The Mn^{2+} specificity of the enhancement of LUV endonuclease activity prompted re-examination of the effect of EDTA on the activity. EDTA (1mM) was found to eliminate the activity which could then be specifically reintroduced by the addition of 2.5mM Mn^{2+} ions; Mg^{2+}, Ca^{2+}, Str^{2+}, Fe^{2+}, Ni^{2+}, Cu^{2+} or Co^{2+} ions having no effect. This does not occur in cruder extracts possibly for the same reason that Mn^{2+} doesn't enhance activity. LUV endonuclease therefore appears to require Mn^{2+} ions for activity.

CHAPTER 4

DISCUSSION

A model of excision repair of DNA damage in *D.radiodurans*

The successful isolation and characterisation of *D.radiodurans* strains deficient in the excision repair of pyrimidine dimers reveals a novel situation, apparently unique to *D.radiodurans*, in which two independent mutations are required to completely eliminate the excision of dimers. One mutation must be in the *mtcA* gene and one in any one of the *uvsC*, *uvsD* or *uvsE* genes. The primary cause of the excision defect in such double mutant strains is the failure to incise UV-irradiated DNA. Strains carrying single mutations in any one of these genes are incision proficient and will excise pyrimidine dimers from DNA in a manner which varies from the wild-type strain but which is as efficient overall since these strains are UV resistant. These observations provide the basis for a model of excision repair in *D.radiodurans*, the fundamental features of which are described below. Other data are discussed in terms of this model.

It is proposed that *D.radiodurans* R_1 possesses two independent excision repair enzymes that incise DNA in response to pyrimidine dimers; UV endonuclease α, the putative product of the *mtcA* and *mtcB* genes and UV endonuclease β, a novel type of UV incision enzyme which is inactivated by mutation in the *uvsC*, *uvsD* and *uvsE* genes. (The term 'UV endonuclease' is used as a convenient term, although the mode of incision is not known). Pyrimidine dimers are substrates for UV endonucleases α and β. However, the manner of incision and subsequent damage removal differs between them such that excision repair *via* UV endonuclease α is separated from excision repair *via* UV endonuclease β ie. *D.radiodurans* has two distinct DNA excision repair pathways.

These are distinguishable with respect to (a) the substrate range of the initiating endonucleases, (b) the rate of subsequent damage removal and repair, (c) the accessibility of the repair sites to an exonuclease that requires protein synthesis, produced in response to DNA damage to moderate its action.

D.radiodurans also contains a third UV endonuclease which is active towards a minor UV-induced DNA photoproduct, probably pyrimidine hydrates, but which is not active towards pyrimidine dimers. The model described above, including other aspects to be described is summarized in figure 58. The characteristics of the UV endonucleases are summarized in Tables 18 and 19.

The substrate range of UV endonucleases α and β and the HUV endonuclease

The loss of 'LUV endonuclease' activity from cell extracts *in vitro* when the *uvsC*, *uvsD* or *uvsE* genes are inactive, but not when the *mtcA* gene is inactive, identifies the 'LUV endonuclease' activity as being solely due to UV endonuclease β. This provides strong support for the existence of two pyrimidine dimer UV endonucleases since the singly mutant strains lacking UV endonuclease β *in vitro* remain incision proficient *in vivo* and are UV resistant. Also, the failure of UV endonuclease α to operate *in vitro* under the same reaction conditions as UV endonuclease β demonstrates a fundamental difference between UV endonucleases α and β.

The presence of UV endonuclease β *in vitro* when the *mtcA* gene is mutated excludes it from a direct role in incision of mitomycin C crosslinks and bromomethylbenzanthracene adducts

Figure 58 Model of excision repair of UV photoproducts in *D.radiodurans*

A. UV irradiation produces pyrimidine dimers, thy(6,4)pyo adducts and pyrimidine hydrates in DNA. Pyrimidine dimers are incised *via* either UV endonuclease α or β, thy(6,4)pyo adducts *via* UV endonuclease α and pyrimidine hydrates *via* the HUV endonuclease. UV also induces an SOS-like response causing derepression of the inducible terminator.

B and C. UV endonuclease α and β repair DNA *via* two different forms of nucleotide excision repair, UV endonuclease α excision repair events being akin to those of the *E.coli* UVRABC enzyme. The HUV endonuclease acts *via* base excision repair. UV endonuclease β requires Mn^{2+} ions, whereas the HUV endonuclease acts independently of all cofactors. UV endonuclease α may act independently of divalent cations. Inducible terminator (■) is synthesised.

D. Post-incision removal of pyrimidine dimers proceeds *via* degradative enzymes (● and ◇) that require divalent cations. The enzyme that responds to inhibition by the inducible terminator acts only at UV endonuclease α excision repair sites.

D. Repair is completed by polymerase (□) and ligase action. The inducible terminator gene becomes repressed as the SOS response reverts to the 'off' state.

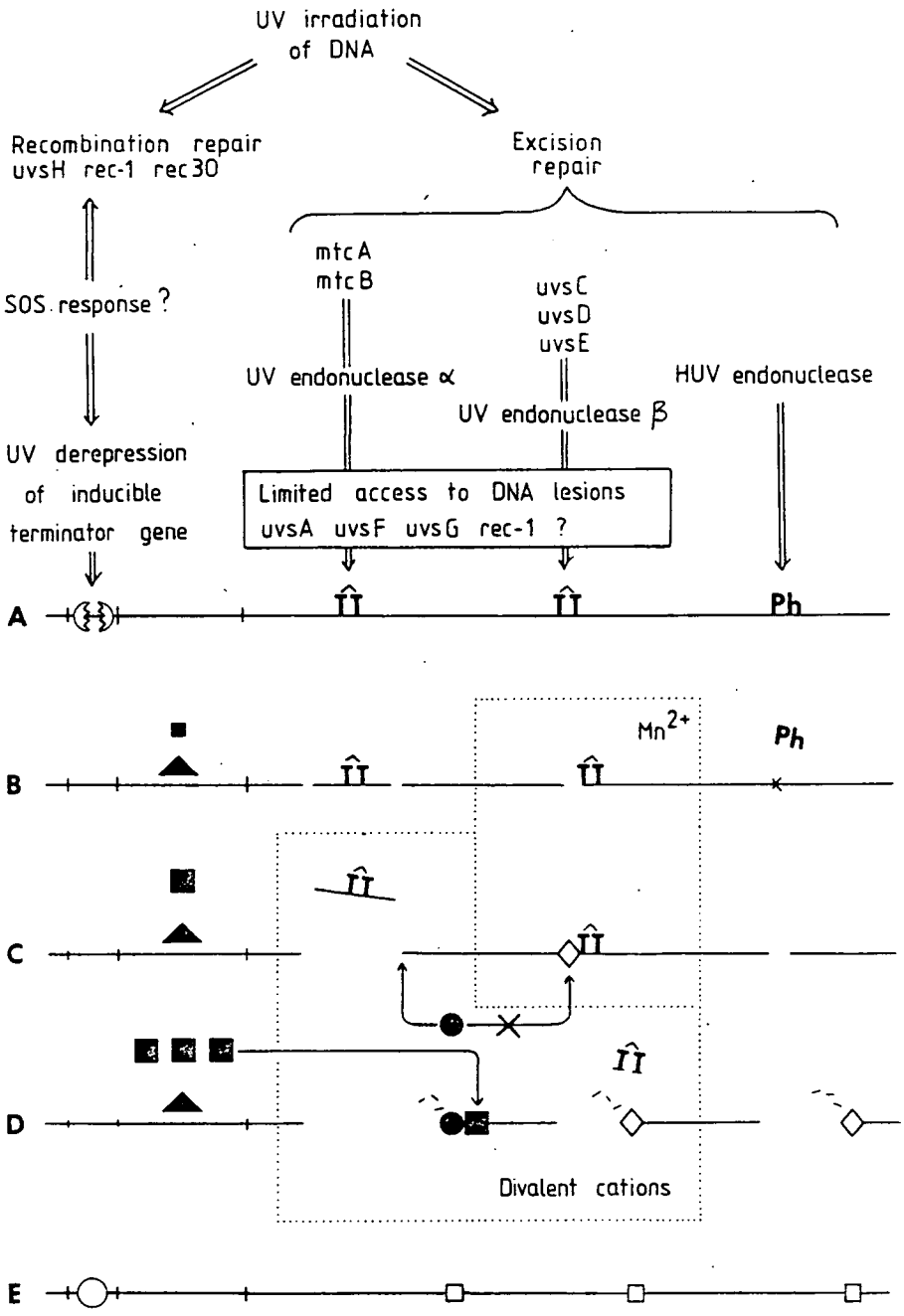

UV irradiation
of DNA
Recombination repair
uvsH rec-1 rec30
Excision
repair
SOS response ?
mtcA
mtcB
uvsC
uvsD
uvsE
UV endonuclease α
UV endonuclease β
HUV endonuclease
UV derepression
of inducible
terminator gene
Limited access to DNA lesions
uvsA uvsF uvsG rec-1 ?
A
Ph
B
Mn2+
Ph
C
D
Divalent cations
E

Table 18

Summary of the relationship between UV endonuclease activities of *D.radiodurans*, strains deficient in excision repair and the requirement for the UV inducible terminator of exonuclease activity.

Strain	Mutant genotype	Excision repair of pyrimidine dimers	Incision of DNA after UV irradiation	Inducible terminator required	UV endonuclease α Constitutive Cation independent [1] also incises BrMBa adducts, mitomycin C crosslinks and other DNA damage	UV endonuclease β Constitutive Cation independent [1] EDTA inactivated [2] NEM inhibited ATP independent 80 – 150 mM ions MW 36000	HUV endonuclease Constitutive Cation independent EDTA no effect [2] NEM independent ATP independent 0 – 200 mM ions MW 27000
Wild type		+	+	+	+	+	+
302	mtc A$^-$	+	+	–	–	+	+
UVS9	mtc A$^-$ uvs C$^-$	–	–	–	–	–	+
UVS25	mtc A$^-$ uvs D$^-$	–	–	–	–	–	+
UVS78	mtc A$^-$ uvs E$^-$	–	–	–	–	–	+
91	uvs C$^-$	±	+	+	+	–	+
251	uvs D$^-$	±	+	+	+	–	+
781	uvs E$^-$	±	+	+	+	–	+
All other repair deficient strains		+	+	?	+ or –	+	+

1 - in vivo 2 - in vitro

Table 19

Comparison of the UV endonuclease activities of *D.radiodurans* with the well-characterized activities from *E.coli*, *M.luteus* and phage T4.

a. Sancar *et al.*, 1983
b. Bonura *et al.*, 1982
c. Murray 1979
d. Rupp *et al.*, 1982
e. Friedberg 1972
f. Has eltine *et al.*, 1980
g. Katcher and Wallace 1983
h. Riazuddin 1980
i. Friedberg *et al.*, 1981
j. Braun and Grossman 1974a
k. Seeberg *et al.*, 1980
ℓ. Fong and Bockrath 1979a
m. Nakayama *et al.*, 1971
n. Friedberg and King 1971
o. Friedberg *et al.*, 1980

Enzyme	*E.coli* UVRABC "exinuclease"	T4 pyrimidine dimer glycosylase	*M.luteus* pyrimidine dimer glycosylase	*D.radiodurans* UV endonuclease α	*D.radiodurans* UV endonuclease β	*D.radiodurans* HUV endonuclease	*E.coli* endonuclease III
genes eliminating incision	*uvrA* a. *uvrB* *uvrC*	*denV* d.	?	*mtcA* *(mtcB?)*	*uvsC* *uvsD* *uvsE*	?	?
Lesions recognised	pyrimidine dimers, MTC cross-links, BrMBa adducts + others c.	pyrimidine dimers e.	pyrimidine dimers f.	pyrimidine dimers MTC cross-links BrMBa adducts + others	pyrimidine dimers, minor MTC lesions HA lesions?	pyrimidine hydrates	pyrimidine hydrates and other fractured pyrimidines g.
Size (daltons)	>250,000 d.	18,500 b,	12,500 f.	?	36,000	27,000	22,000 g.
Divalent cation requirement	Mg^{2+} a.	None b.	None f.	None ?	Mn^{2+}	None	None g.
ATP requirement	Yes a.	No b.	No f.	?	No	No	No g.
Operative in caffeine (25mM)	No. k. ℓ.	Yes n.	?	?	Yes	Yes	?
Operative in acriflavin ($1\mu gml^{-1}$)	No k. ℓ.	Yes n.	Yes m.	?	Yes	Yes	?
Disulphide bonds required for activity	No? k.a.	No i.	No h.	?	No	No	No g.
Sulfide bonds in active sites	Yes k.	Yes i.	No h.	?	Yes	No	Yes g.
Freeze stable	?	No i.	Yes h.	?	Yes	Yes	?
pH range	?	Wide i. (>6→8) opt ∿ 7.2	Wide h. (>6→8) opt ∿ 7.5	?	wide (>6→8)	wide (>6→8)	wide g. (>6→8) opt ∿ 7.2
Preferred ionic conc.	100mM o.	100mM i.	100mM j.h.	?	100mM	100mM	100mM g.

in vivo since inactivation of the *mtcA* gene alone eliminates DNA incisions in response to these lesions *in vivo* (Tempest and Moseley 1980; Kitayama *et al.*, 1983). Mutation of the *mtcB* gene produces an identical phenotype to that produced by mutation of *mtcA* (Moseley and Copland 1978) suggesting that both may code for subunits of UV endonuclease α. Inactivation of UV endonuclease β also renders cells slightly more sensitive to mitomycin C when UV endonuclease α is functional. This is a small effect relative to the sensitivity induced by inactivation of UV endonuclease α and may be the result of a failure of UV endonuclease β to recognise a minor mitomycin C lesion that is only present in significant amounts after exposure to high doses of mitomycin C. Strains lacking UV endonuclease α die below this dose, hence the lack of effect of mutations in *uvsC*, *uvsD* and *uvsE* on mitomycin C survival when the *mtcA* gene is inactive.

Neither UV endonuclease α or β is apparently involved in the repair of lethal DNA alkylation damage since loss of either or both excision enzymes has no effect on survival in MMS or incision in response to MMS, EMS, ENNG or MNNG *in vivo*. However, the *mtcA* gene and hence UV endonuclease α is known to be required for removal of a premutagenic DNA lesion, probably O^6 methylguanine, induced by MNNG (Tempest and Moseley 1980). The majority of alkylation products induced in *D.radiodurans* DNA are therefore likely to be repaired *via* a range of DNA glycosylases, as in *E.coli* that are distinct from UV endonucleases α and β since the latter are not responsible for removal of lethal alkylated nucleotides. The endonuclease activities against MMS or EMS treated DNA observed *in vitro*

are likely to be examples of such glycosylases ie. acting in concert with AP endonucleases possibly including the major AP endonuclease activity also observed *in vitro*.

Either UV endonuclease α or β may also be involved in incision in response to HA induced DNA damage since there is a reduction in the degree of incision in response to HA in strains lacking both UV endonucleases. A reduction in incision in response to HA in the absence of UV endonuclease α would not be surprising since its loss induces sensitivity to the lethal effects of HA (Tempest and Moseley 1978).

UV endonuclease α therefore appears to have a substrate range that is as broad as that of the *E.coli* UVRABC enzyme whereas UV endonuclease β has a narrower substrate range, largely restricted to pyrimidine dimers in a manner reminiscent of the T4 and *M.luteus* UV glycosylases (see introduction, Table 19). However, these enzymes are highly specific for pyrimidine dimers whereas UV endonuclease β may recognise other lesions as well.

Pyrimidine dimers appear to be substrates for UV endonuclease β *in vivo* because the minimum UV dose which causes 1 μg of ccc DNA to be incised in an excess of enzyme would be expected to produce only 2-3 pyrimidine dimers on average per plasmid molecule of this size (Harm 1981; Seawell and Ganesan 1981). This minimum dose is only 1.4 fold greater than for the *M.luteus* pyrimidine dimer glycosylase acting on pML2 and is comparable to that found for the T4 UV glycosylase on a plasmid of similar size (Seawell and Ganesan 1981). The next most frequent UV photoproducts in *E.coli* DNA at this UV dose are pyrimidine hydrates and thy(6,4)pyo adducts at 0.1-0.2 lesions per plasmid molecule of the size of pML2 (Patrick and Rahn 1976).

The third UV endonuclease, the HUV endonuclease, was observed in the absence of UV endonucleases α and β and is therefore distinct from these. The HUV endonuclease also has a smaller molecular weight (27000) than UV endonuclease β (36000) and is unlike UV endonuclease β in being active in EDTA, NEM, acriflavin and low salt concentrations. It also fails to incise lightly irradiated DNA *in vitro* or to promote excision of pyrimidine dimers *in vivo* and may be partially responsible for the DNA incision seen after high UV doses *in vivo* in strains lacking UV endonucleases α and β (figure 19). The HUV endonuclease substrate is approximately 20 fold less frequent than the substrate of UV endonuclease β which is approximately the expected frequency of thy(6,4)pyo adducts or pyrimidine hydrates in DNA. Several points favour the substrate being pyrimidine hydrates. Firstly, these lesions are produced by OsO_4 treatment of DNA, and an activity was identified which copurified with the activity towards UV-irradiated DNA. The two activities have indistinguishable molecular weights and reaction properties. Secondly, thy(6,4)pyo adducts would be expected to be excised as "bulky" lesions by a broad spectrum enzyme as is the case in *E.coli* (Sancar *et al.*, 1983) ie. UV endonuclease α, whereas pyrimidine hydrates in other bacteria are excised by small cation-independent enzymes such as endonuclease III (Katcher and Wallace 1983). The HUV endonuclease is a small, cation independent enzyme. Finally, the difference in minimum UV doses to complete incision by UV endonuclease β and the HUV endonuclease is exactly the same as that between the *M.luteus* UV glycosylase and *M.luteus* pyrimidine hydrate glycosylase (Riazzudin 1980). The HUV endonuclease may therefore be a

glycosylase similar to endonuclease III of *E.coli* and initiate the previously observed excision of pyrimidine hydrates from *D.radiodurans* DNA (Targovnik and Hariharan 1980).

On the nature of UV endonuclease β and the *uvsC*, *uvsD* and *uvsE* gene products

UV endonuclease β is distinct from the three other enzyme systems known to incise DNA in response to pyrimidine dimers primarily by virtue of its intermediate molecular weight and its Mn^{2+} requirement (see introduction, Table 19). Inactivation of the *uvsC*, *uvsD* or *uvsE* genes, which eliminates UV endonuclease β, cannot be comparable to inactivation of the *uvrA*, *uvrB*, or *uvrC* genes in *E.coli* because UV endonuclease β is in total smaller than any one of the UVRA, UVRB or UVRC proteins. The small size of UV endonuclease β and the fact that it can be purified by column chromatography without a complementation assay suggests that it is a single protein possibly produced by one of the *uvsC*, *uvsD* or *uvsE* genes and is therefore similar to the two known pyrimidine dimer glycosylases. This is also supported by the inability of mixtures of extracts from strains mutant in *uvsC*, *uvsD* or *uvsE* to complement the loss of UV endonuclease β. The effect of inactivation of each of the genes on the majority of aspects of repair studied here is generally *uvsE>uvsC>uvsD* (ie. inactivation of *uvsE* has the greatest effect). These differences could be an effect of variation in UV endonuclease β synthesis. Two of the *uvsC*, *uvsD* and *uvsE* genes may therefore be regulatory, perhaps in a manner similar to the control of glycosylase action within the adaptive response by the *alk* gene (Lindahl 1982). However, UV endonuclease β,

which is apparently synthesised constitutively, is completely absent *in vivo* and *in vitro* when each of these genes is mutant so that control would need to be positive and very efficient. Alternatively, because the size of UV endonuclease β is approximately twice that of the T4 and *M.luteus* pyrimidine dimer glycosylases the enzyme may be a composite of two or more different smaller proteins or a single protein dimer as in the case of endonuclease III (Katcher and Wallace 1983). Unfortunately the size of the protein products of the *uvsC*, *uvsD* and *uvsE* genes could not be determined from SDS polyacrylamide gels due to there being more than one difference in the pattern of protein bands in strains mutant in each of these genes. The range of differences observed in polyacrylamide gels, both when gels were stained and in DNA containing gels, indicates that each of the strains mutant in *uvsC*, *uvsD*, *uvsE* and/or *mtcA* contain a range of additional mutations that were introduced by the initial exposure to MNNG.

In view of the above and the ATP independence of UV endonuclease β the enzyme may be a unique (Mn^{2+} requiring) pyrimidine dimer glycosylase. This is supported by the relative insensitivity of the enzyme to caffeine and acriflavin, a characteristic of the pyrimidine dimer UV glycosylases (Friedberg and King 1971; Nakayama *et al.*, 1971) but not the UVRABC enzyme (Seeberg *et al.*, 1980; Fong and Bockrath 1979a). However, no glycosylase action was detected by the assay used here indicating that the enzyme incisions are not due to glycosylase action and may therefore be due to true endonuclease action.

Incision by UV endonucleases α and β *in vivo*

Both UV endonucleases α and β appear to operate *in vivo*

in the presence of EDTA which is surprising particularly since UV endonuclease β requires Mn^{2+} ions *in vitro*. However, this could be the result of the high Mn^{2+} concentration around *D.radiodurans* DNA which is 100x that found in other bacteria such as *E.coli* (Leibowitz *et al.*, 1976) and may locally exceed the chelating capacity of the EDTA. Also, although EDTA in growth medium inhibits other excision processes and a non-specific intracellular butanol-inducible endonuclease (Driedger and Grayston 1970), the EDTA may not actually enter the cell through the extensive and complex cell envelope; rather intracellular ions would be withdrawn. Since Mn^{2+} may be bound by UV endonuclease β it may be necessary for EDTA to be in intimate contact with the enzyme to extract the Mn^{2+} ions. This obviously could not occur if the EDTA does not enter the cell, and raises the possibility that UV endonuclease α is also either Mn^{2+} requiring and/or binds a cation thereby remaining active *in vitro*.

UV endonuclease α, in addition to UV endonuclease β appears to be constitutive , or at least present in functionally adequate amounts to cope with large amounts of damage, since inhibition of protein synthesis does not reduce *in vivo* incision by either enzyme and UV endonuclease β does not require induction to isolate large quantities *in vitro*.

The possible existence of more than one pyrimidine dimer UV incision system in other organisms

Although the work presented here provides the first direct evidence of an organism possessing two enzymes capable of incising DNA in response to pyrimidine dimers, it is possible that other organisms also possess a UVRABC type enzyme (UV

endonuclease α in *D.radiodurans*) and also a dimer-specific enzyme (UV endonuclease β in *D.radiodurans*). However, these may not be as functionally equal in excision repair as in *D.radiodurans*. For instance in *B.subtilis* the main excision repair system has a broad substrate range similar to nucleotide excision repair in *E.coli* (Friedman and Yasbin 1983). However, *B.subtilis* also possesses an inducible excision repair system responsible for Weigle reactivation which is specific for pyrimidine dimers in DNA (Fields and Yasbin 1983; Hadden 1979a, 1979b). The existence of two such enzymes in *M.luteus* would generally explain much of the confusing work on repair-deficient *M.luteus* mutants as described in detail in the introduction. It would also help to resolve the similarly complicated array of repair-deficient phenotypes which can be created in *H.influenzae* e.g. the DB115 strain which is Hcr^-, but excision repair proficient (Setlow *et al.*, 1968). A component of excision repair in *E.coli* now also appears to act as a pyrimidine dimer glycosylase (Bonura *et al.*, 1982; Radany and Friedberg 1982) indicating that *D.radiodurans* may differ from these other organisms only in the control and efficiency of these two processes.

Control of DNA incision in response to pyrimidine dimers

The number of DNA incisions made in wild type *D.radiodurans* after UV irradiation does not exceed about 2% of the number of pyrimidine dimers in the DNA; in *E.coli* the figure is approx 3% (Setlow 1967b). The excision of lesions in both organisms is therefore coordinated and sequential, ie. new excision repair sites are begun after others are completed. The mechanism of

this coordinated control in *E.coli* is not known but is possibly the result of limiting quantities of UVRC protein since there may be as few as 10 molecules per cell (Yoakum and Grossman 1981). The increase in the number of DNA incisions in proportion to the number of pyrimidine dimers whilst maintaining the fraction of dimers incised at an instant at around 2-3% indicates the presence of an incision capability that is not utilised at lower UV doses. Increasing UV doses may possibly increase the number of endonuclease molecules *via* SOS induction in *E.coli* or *via* a comparable response in *D.radiodurans*. However, the number of breaks introduced for a given number of dimers in DNA in *D.radiodurans* is not reduced by the absence of either UV endonuclease α or β suggesting that the number of incisions may be limited by other factors such as the accessibility of the DNA lesions. The excess of DNA incisions produced when the *uvsA*, *uvsF*, *uvsG* and *rec-1* genes are mutant suggests that these could have roles in control of access of endonuclease to DNA. The phenotypes produced by these mutations are largely consistent with those produced by polymerase or ligase mutations in *E.coli* both of which cause excessive DNA degradation and accumulation of DNA breaks (Boyle *et al.*, 1970b; Pauling and Hamm, 1968) as new excision sites are begun but not completed (Seeberg and Strike 1976; Seeberg *et al.*, 1980). However, the excessive number of single-strand breaks produced when *uvsA*, *uvsF*, *uvsG* or *rec-1* are mutant would have to have accumulated during the period in phosphate EDTA buffer up to lysis on alkaline sucrose gradients. This seems unlikely because a similar accumulation does not occur during this period in the wild type where post-incision repair events are blocked by EDTA and phosphate buffer, ie. the excess

incisions in *uvsA*, *uvsF*, *uvsG* and *rec-1* mutants appear not to accumulate but to appear immediately after UV irradiation. The failure of the wild type to accumulate incisions when post-incision mechanisms are blocked would result if new excision sites cannot be started until those already under repair have been completed. Mutation of *uvsA*, *uvsG*, *uvsF* or *rec-1* may therefore disable the sequential control of excision repair rendering all dimers immediately and simultaneously available for incision by an excess of incision enzymes. An active control of excision would not be surprising since *D.radiodurans* can survive one lesion every 150 nucleotides and this would probably result in lethal fragmentation of the chromosomes if all these lesions were to lead to incision simultaneously. Indeed the quantity of DNA degraded per Jm^{-2} UV in the strains that 'overincise' DNA after UV irradiation is greater than in the wild type implying that the additional incisions cannot be dealt with by post-incision mechanisms.

Post-incision repair of DNA damage

The rate of pyrimidine dimer excision in strains where only UV endonuclease α is absent is similar to that of the wild type whereas excision is slowed more markedly by the loss of UV endonuclease β and no excision occurs when both are absent. This situation is mirrored with respect to the rates of post-UV irradiation, rates of DNA degradation, rates of recovery in split UV dose experiments, the resealing of UV-induced excision breaks and the rate of resumption of DNA synthesis after UV irradiation. In each case an absence of UV endonuclease α has less effect than an absence of UV endonuclease β, whereas an

absence of both causes the most profound inhibition of each parameter. In view of the constant number of incisions made in response to a particular UV dose in the absence of either UV endonuclease α or β it would seem that the post-incision removal of DNA damage *via* UV endonuclease α is a slower process than *via* UV endonuclease β and that the two processes are distinct. The independence of each excision pathway is demonstrated by the dependence of the component of UV irradiation (or Mitomycin C) induced DNA degradation that requires protein synthesis to moderate its action at excision repair sites, on the presence of UV endonuclease α. It is envisaged that an inducible protein (an "inducible terminator") is synthesised as part of an SOS-like response and that a role of this protein is to attenuate the action of an exonuclease. The inducible protein may also be required to regulate DNA degradation after X-irradiation which is also enhanced if protein synthesis is blocked (Dean *et al.*, 1970) and may be one of the damage inducible proteins α,β,γ or δ identified by Hansen (1980). A simple explanation for the loss of UV inducible DNA degradation along with UV endonuclease α is that the exonuclease which requires the inducible terminator fails to act at UV endonuclease β incisions ie. the manner of incision by UV endonucleases α and β is different and this defines the manner of subsequent damage removal.

A similar mechanism for post-irradiation control of exonuclease activity by an 'inducible terminator' occurs in *E.coli* where the *recA* protein, induced as part of the SOS response, directly prevents the *recBC* exonuclease from completely degrading the chromosome by binding to DNA in its path (Pollard and Randall 1973; Pollard and Achey 1975; Gudas and Pardee 1975;

Dharmalingham and Goldberg 1980; Prell and Wackernagel 1981; Williams *et al.*, 1981). However, the *E.coli* recBC exonuclease is probably not involved in excision repair and appears to act at regions other than excision sites (Waldstein *et al.*, 1974; Masker *et al.*, 1978; Fong and Bockrath 1977). *D.radiodurans* may synthesise further proteins required for the resealing of single-strand breaks and recovery of viability in the absence of UV endonuclease α as part of an SOS response, since protein synthesis is required to complete repair of single-strand breaks in the absence of UV endonuclease α but is not required to prevent excessive DNA degradation.

The enhancement of post-irradiation lethality in *D.radiodurans* by prevention of protein synthesis occurs in parallel with enhanced DNA degradation. The selection of a strain mutant in the *mtcA* gene *via* resistance to this enhanced lethality confirms that UV endonuclease α incisions are the site of action of the DNA degradation and hence the lethality. 40% of DNA is degraded for a 90% reduction in survival in the wild type strain in the absence of protein synthesis which suggests that the DNA degradation probably does not occur in all cells and is similar to the 'all or nothing' DNA degradation observed under similar circumstances in *E.coli* where some cells contain no DNA whereas the DNA of others remains undegraded (Pollard and Randall 1973). In support of this, the *D.radiodurans* wild type strain can complete repair of a proportion of UV induced single-strand breaks in the absence of protein synthesis which would probably not occur if 40% of each chromosome was degraded.

DNA degradation in the presence of protein synthesis in

D.radiodurans is largely the product of excision repair by UV endonuclease α and β in a similar manner to the dependence of the majority of post-irradiation degradation on functional *uvrA* and *uvrB* genes in *E.coli* (Setlow 1968). Division of the total DNA degradation attributable to excision by the number of lesions originally present in DNA gives an estimate of about 20 nucleotides removed per pyrimidine dimer in wild-type *D.radiodurans* or in the absence of either UV endonuclease. This is near the 13 nucleotides removed in nucleotide excision repair (Sancar *et al.*, 1983) and considerably more than the 4-7 excised after T4 or *M.luteus* UV glycosylase mediated incisions (Bonura *et al.*, 1982; Grossman *et al.*, 1968). However, the proportion of this figure that arises from a typical *D.radiodurans* excision site is unknown, since the figure is an average from a population of repair events that may contain a proportion of long patches.

The residual dose-independent DNA degradation observed in the absence of UV endonucleases α and β is similar to that observed in *uvrA* mutants of *E.coli* caused by random degradation partly by the recBC enzyme, and which does not depend on DNA breaks (Suzuki *et al.*, 1966; McGrath *et al.*, 1966; Setlow 1967a; Fong and Bockrath 1979b). The excision of pyrimidine hydrates and other similar photoproducts *via* the HUV endonuclease may also contribute to this degradation.

The post-incision removal of damage in *D.radiodurans* is eliminated by EDTA and therefore requires divalent cations whereas incision by either UV endonuclease occurs in the presence of EDTA. The two processes may therefore be distinct as in *M.luteus* where the damage removal after ion-independent incisions is apparently carried out by ion-requiring exonucleases (Kaplan *et al.*, 1971). However, since the *uvrD* DNA helicase II

implicated in nucleotide excision repair also requires divalent cations (Abdel-Monem *et al.*, 1977a) it may be that a helicase is also being inhibited by EDTA in *D.radiodurans*. Unfortunately the divalent cations required by the damage removal mechanisms could not be identified here.

Linkage between *mtcA*, *uvsF* and *uvsG*

Double transformants to UV resistance and mitomycin C resistance were obtained from strains mutant in *mtcA* and *uvrF* or mutant in *mtcA* and *uvsG* but not from those mutant in *mtcA* and *uvsA* suggesting that *mtcA* is closely linked to *uvsF* and *uvsG* but not *uvsA*. The *uvsF* gene is closer to *mtcA* than *uvsG* although the order of the genes is not known due to the inability to obtain strains carrying mutations in both *uvsG* and *uvsF*. The relationship between linkage value and true molecular separation is unknown but assuming the integrated region is similar to that in other bacteria eg. $6x10^6$ daltons in *Haemophilus spp* (Notani and Goodal 1966), $2x10^6$ daltons in *Pneumococcus spp* (Gurney and Fox 1968) and $3x10^6$ daltons in *B.subtilis* (Dubnau and Cirigliano 1972), an average of 12 kilobases, then the distance between the linked genes is less than 0.2% of the chromosome.

Repair genes and mutation

None of the repair deficient strains examined was UV mutable. The residual repair of UV damage in these strains was therefore error-free, supporting the notion that error-prone repair is absent from *D.radiodurans* (Tempest and Moseley 1982). None of the mutations in the repair

genes examined, except *mtcA*, had any effect on mutagenesis *via* MMS, and therefore they do not interrupt the removal of mutagenic lesions produced by alkylating agents. The *uvsA*, *uvsG*, *uvsF*, *uvsH* and *rec-1* genes are, however, involved in repair of lethal MMS-induced lesions. This involvement is indicative of a polymerase or ligase (Pauling and Hamm 1968; Boyle *et al.*, 1970). Alternatively if these genes are involved in restricting access to DNA lesions then the implication is that the access of the enzymes that lead to incision in response to MMS damage are also restricted in the wild type.

On the DNA degradation produced by mutation of the *uvsA*, *uvsF*, *uvsG* and *rec-1* genes

The excessive post-irradiation DNA degradation produced by mutation of the *uvsA*, *uvsF*, *uvsG* and *rec-1* genes is a likely consequence of the increased frequency of DNA single-strand breaks caused by those mutations and also the likely cause of the failure to repair these breaks. An excess of DNA single-strand breaks has a similar effect in *E.coli* (Pauling and Hamm 1968). EDTA or phosphate buffer reduces the excessive degradation in *D.radiodurans*, but not the excessive incision, suggesting that the excessive degradation is a consequence of the excessive incision and not *vice versa*. Mutation of these genes also enhances MMS-induced DNA degradation but surprisingly not mitomycin C-induced DNA degradation. UV endonuclease α is responsible for repair of the majority of mitomycin C DNA damage suggesting that this excision route is unaffected by the *uvsA*, *uvsF*, *uvsG* or *rec-1* genes; these genes exert their effect

solely on UV endonuclease β which would therefore be the source of the UV induced excessive DNA incision and DNA degradation when these genes are mutant. Proof of this could be obtained by construction of strains lacking UV endonuclease β and one of the *uvsA*, *uvsF*, *uvsG* or *rec-1* genes. Unfortunately this cannot be achieved at present due to the lack of suitable selectable phenotypes. However, some support for this is provided by the lack of effect of the presence or absence of UV endonuclease α on the excessive post-UV DNA incision and degradation produced when *uvsA*, *uvsG*, *uvsF* or *rec-1* are mutant. Also, only a slight further enhancement of post-UV DNA degradation occurs in the absence of protein synthesis and this also occurs independently of UV endonuclease α. Since an active UV endonuclease α can produce an equivalent excess of post-UV DNA degradation in the absence of protein synthesis, the removal of this component along with UV endonuclease α may be expected to reduce the excessive degradation. The fact that this doesn't occur may be because UV endonuclease α is not the source of the excess degradation.

DNA synthesis blocks in repair deficient strains

The severity of the effect of UV on DNA synthesis in *D.radiodurans* reflects the decreasing excision-repair capability of particular strains when the *uvsA*, *uvsG*, *uvsF* and *rec-1* genes are wild type. This is presumably due to the persistence of pyrimidine dimers in DNA, the cause of similar DNA synthesis delays in excision repair deficient *E.coli* (Setlow 1968), *H.influenzae* (Modak and Setlow 1969) and *M.luteus* (Mahler *et al.*, 1971). However, thy(6,4)pyo adducts, pyrimidine hydrates and

AP sites (generated by glycosylase action) also block DNA replication (Strauss *et al.*, 1982) and may contribute to the replication blocks in *D.radiodurans*. The residual DNA synthesis in excision-deficient strains may be analogous to 'stable DNA synthesis' in *E.coli* (Kogoma *et al.*, 1979) which is a combination of the bypassing pyrimidine dimers by the replication complex (Rupp and Howard-Flanders 1968; Iyer and Rupp 1975; Moore *et al.*, 1981) and the resynthesis stages of recombinational repair events (West *et al.*, 1982) but is not error-prone synthesis over dimers (Strauss *et al.*, 1982; Lackey *et al.*, 1982) since excision repair-deficient *D.radiodurans* strains are immutable by UV (see results, Moseley and Evans 1983). Protein synthesis is required for DNA synthesis to recover probably as a result of its necessity for prevention of excessive DNA degradation. In the absence of protein synthesis the degraded regions that are not resealed block DNA synthesis.

Mutations in any one of the *uvsA*, *uvsG*, *uvsF* or *rec-1* genes causes a profound block to DNA synthesis despite pyrimidine dimers being removed from the DNA. In view of this and the excessive incision and resultant DNA degradation in these strains, the single-stranded DNA regions generated by the degradation are the likely cause of the replication blocks since these are known to block DNA replication in *E.coli* (Trgovcevic *et al.*, 1983; Hofemeister *et al.*, 1980).

The site of the enhancement of UV induced lethality by caffeine

Caffeine increases the lethal and mutagenic effects of UV light in other bacteria (Witkin 1969) by interfering with excision repair (Clark 1967; Sideropolos and Shankel 1968). It also

has an anti-mutagenic effect in excision-deficient strains due to suppression of an unidentified component of post-replication repair (Clark 1967; Ichikawa-Ryo and Kondo 1980). Caffeine inhibits incision by the UVRABC enzyme (Seeberg *et al.*, 1976; Seeberg *et al.*, 1980; Fong and Bockrath 1979a) but not by pyrimidine dimer glycosylases (Friedberg and King 1971). The lethal effect of caffeine in *D.radiodurans* is not due to inhibition of UV endonuclease β or any other parameter of excision repair since it has no effect of the rate of excision of pyrimidine dimers and the rate of recovery from UV damage. The source of the lethality is in DNA replication since DNA synthesis is inhibited by caffeine and this is enhanced by UV irradiation. Any DNA polymerases involved in excision repair in *D.radiodurans* are therefore distinct from those involved in DNA replication. *D.radiodurans* contains three DNA polymerases (Kitayama and Matsuyama 1977; Kitayama *et al.*, 1978), one of which may be similar to the excision repair DNA polymerase 1 in *E.coli* (Kitayama *et al.*, 1978; Sancar *et al.*, 1983) the polymerase activity of which is caffeine insensitive (Solberg *et al.*, 1978). Other polymerases involved in replication may be inactivated directly or as the result of the binding of caffeine to DNA or due to the intercalation of caffeine into DNA which causes denaturation, base destacking and DNA unwinding (Ts'o *et al.*, 1982; Ts'o and Lu 1964; Ts'o 1974). Pyrimidine dimers also cause local DNA denaturation creating single-stranded regions that strongly bind caffeine which may be why UV exacerbates the inhibitory effect of caffeine on DNA replication (Kelly *et al.*, 1969a; Ts'o 1974).

On the nature of the *uvsH* gene

The *uvsH* gene is involved in repair of UV, mitomycin C and MMS induced lethal damage *via* a pathway which acts largely independently of excision repair of pyrimidine dimers. This is likely to be in the recombination pathway for repair of DNA identified by Moseley and Copland (1972) that repairs all three forms of DNA damage. Inactivation of this pathway *via* the rec30 mutation produces a similar phenotype to that produced by mutation of *uvsH* except that rec30 abolishes transformation (Moseley and Copland 1975). In *E.coli* a *recBC* mutation produces a *uvsH*-like phenotype (Telander *et al.*, 1981; Barbour and Clark 1970) suggesting a similar defect in *D.radiodurans*. However, interspecies comparisons of this type are particularly weak in the case of *uvsH* (and also *rec-1*) since the phenotypes of the strains prevented removal of the *mtcA* mutation by transformation allowing study of *rec-1* and *uvsH* mutations in isolation.

On the nature of the *rec-1* gene

Mutation in *rec-1* produces the most puzzling phenotype of the strains examined. It has features in common with a recombination mutation in eliminating transformation, its 'reckless' DNA degradation and its excision proficiency. However, it also shares features with the phenotypes produced by mutation of the *uvsA*, *uvsF* and *uvsG* genes ie. excessive incision, although the ability of the strain mutant in *rec-1* to repair single-strand breaks distinguishes it from the *uvsA*, *uvsG* and *uvsF* genes. This latter feature is the probable source of the relative UV resistance of the strain. If the source of the additional

incisions is in release of an incision control function, then the *rec-1* gene may have an indirect role in repair generally as does the *recA* gene in SOS induction of a variety of responses. Unfortunately there is no way of determining whether a particular phenotype is the result of multiple mutations in *D.radiodurans* at present which further complicates interpretation of the data on *rec-1*. Further work will therefore be required to untangle this phenotype.

Recombination repair of UV damage

Removal of both excision repair systems from *D.radiodurans* does not prevent DNA repair of UV photoproducts since such strains can be as resistant to UV irradiation as *E.coli* K12. This repair is slower than excision repair, does not involve detectable numbers of single-strand breaks and requires protein synthesis to occur in a similar manner to recombinational repair events in *E.coli* (Smith *et al.*, 1978) suggesting that this is the mechanism operating under these conditions. There is no physical evidence for recombination in *D.radiodurans* partly because of the anomalous incorporation of radioactive and density labels into DNA (see results, Tempest, P.R. 1978), but it would appear that the contribution that this makes to the UV resistance of *D.radiodurans* may be considerable if recombinational repair is the sole source of the residual repair in excisionless strains and successfully repairs (or circumvents) about 700 pyrimidine dimers per genome. This may be a more efficient process than in haploid bacteria such as *E.coli* because of the utilisation of the comparatively vast reservoir of up to 5 genomes per nucleus (Moseley and Evans 1981).

Repair of transforming DNA

The attempts to show that excision repair of UV damaged transforming DNA could occur in *D.radiodurans* were only partially successful. The frequency of transformants obtained using UV irradiated DNA decreased by a factor of 14 in strain UVS78 that lacks cell excision repair, when compared with the wild type. However strains lacking either single UV endonuclease showed a greater reduction, a situation that is difficult to reconcile since it suggests that the presence of one excision repair pathway is more detrimental to repair of the transforming DNA than having no excision repair. The results do show however that excision repair does contribute to the repair of UV irradiated transforming DNA.

Non-specific nucleases

D.radiodurans strains are easily distinguished from the other *Deinococcus spp* by the excessive amount of exogenous nuclease secreted into the growth medium. This nuclease activity may consist of more than one enzyme because mutants deficient in extracellular degradation of DNA still produced wild type levels of non-specific endonuclease activity and also because the endonuclease activity preferred Mn^{2+} ions whereas the degradation of linear DNA was most stimulated by Mg^{2+} ions and may therefore have been due to the action of a distinct exonuclease. The exonuclease activity missing from the *nuc*$^-$ strains has an approximate molecular weight of 220,000 and appears to be able to bind to DNA in the presence of SDS which produces streaks in DNA containing polyacrilamide gels.

The exogenous DNase activities are not involved in DNA repair ie. are not repair enzymes leaking out of the cell since excision repair deficient strains lacking all UV endonuclease activity have normal extracellular DNase activity, the activity is not specific for UV irradiated DNA and is absent from within the wild type cell. The activity is distinct from an exonuclease released into the growth medium by *D.radiodurans* upon X-irradiation (Gentner and Mitchel 1975) since it is excreted constantly.

Intracellular DNase can be separated into thirteen different bands in polyacrilamide gels according to their molecular weight, their divalent cation preferences and their preference for native or single-stranded DNA. Any of those that required divalent-cations especially Mn^{2+} may have been involved in post-incision damage removal.

A possible explanation for the lack of requirement for the inducible terminator in strains mutant in *mtcA* is that they lacked the exonuclease that was moderated by the inducible terminator. However, no single band in DNA containing polyacrilamide gels could be correlated with a functional *mtcA* allele, instead several bands of DNase activity were apparently less active in certain repair deficient strains, particularly bands B and C. On no occasion could an equal distribution of banding be obtained for these strains indicating that the variation was true although it is difficult to reconcile these results with the model of excision repair described earlier.

The major AP endonuclease of *D.radiodurans*

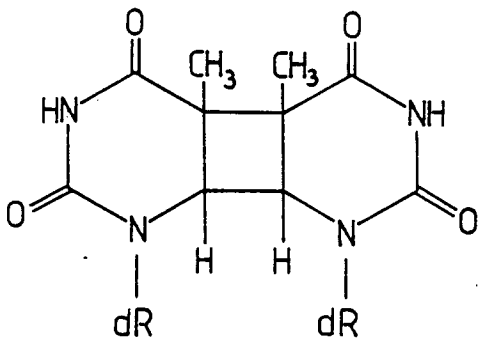
The AP endonuclease identified here is unusual in belonging

to a minor class of EDTA-independent AP endonucleases and distinguishes *D.radiodurans* from *E.coli* and *M.luteus* where the major cell AP endonucleases require divalent cations (Friedberg *et al.*, 1981). The AP endonuclease appears distinct from UV endonucleases α and β ie. is not within the same polypeptide since it is present in strains lacking both of these enzymes. It is also separable and therefore distinct from the HUV endonuclease.

Appendix: The major UV-induced DNA lesions

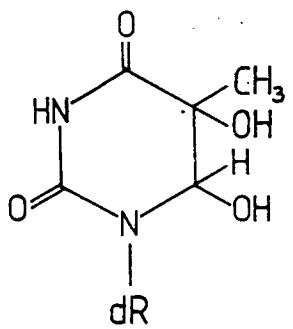
1. Pyrimidine cyclobutane dimers

eg. thymine-thymine dimer



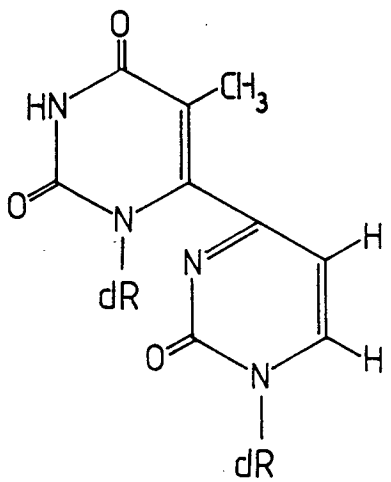
2. Pyrimidine hydrates

eg. 5,6 dihyroxy 5,6 dihyrothymine



3. Pyrimidine 6,4 dimers

eg. 6,4 [pyrimidin-2'one] pyrimidine
(thy 6,4 pyo)



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Isolation and Properties of Strains of *Micrococcus* (*Deinococcus*) *radiodurans* Unable to Excise Ultraviolet Light-induced Pyrimidine Dimers from DNA: Evidence for Two Excision Pathways

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A mutant of *Deinococcus* (formerly *Micrococcus*) *radiodurans* (strain 302, mutant in *mtcA*) sensitive to both the lethal effect of mitomycin C and the mutagenic effect of simple alkylating agents, but having wild-type resistance to UV light, was treated with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in an attempt to isolate strains deficient in the ability to excise UV-induced pyrimidine dimers. Three strains were isolated that were UV-sensitive, but had wild-type resistance to the lethal effect of methyl methanesulphonate and all were shown to be unable to excise pyrimidine dimers. The three strains UVS9, UVS25 and UVS78 had, in addition to the mutation in *mtcA*, mutations in loci designated *uvsC*, *uvsD* and *uvsE*, respectively. When the mutant *mtcA* gene was replaced by its wild-type allele in all three strains they became UV- and mitomycin C-resistant. On incubating the double mutants UVS9, UVS25 and UVS78 with wild-type DNA about 50% of the transformants selected for UV resistance were mitomycin C-sensitive and about 50% resistant depending on whether the mutant *mtcA* or the *uvsC*, *D* or *E* genes had been replaced by their wild-type alleles. Although strains mutant singly in *uvsC*, *D* or *E* were UV-resistant the rates of excision of pyrimidine dimers differed between them and was slower in all of them than in the wild-type and strain 302. The results indicate that wild-type *D. radiodurans* possesses two pathways for the excision of pyrimidine dimers and that mutational blocks in both must exist for the excisionless phenotype to be expressed.

INTRODUCTION

Deinococcus radiodurans (formerly *Micrococcus radiodurans*; Brooks & Murray, 1981) is the type species of a small number of exceptionally radiation resistant, red-pigmented cocci considered to comprise one of the eight major groups of the Eubacteria (Fox *et al.*, 1980). Only two mechanisms for the repair of UV light-damaged DNA in *D. radiodurans* have been described, namely excision and recombination repair (Boling & Setlow, 1966; Moseley *et al.*, 1972; Moseley & Copland, 1975). However, in spite of the isolation of a number of UV-sensitive mutants of *D. radiodurans* from populations of the wild-type treated with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) none has been defective in the excision of UV-induced pyrimidine dimers (Moseley, 1967, 1983; Moseley & Copland, 1975, 1978), an unexpected result since such mutants were the first UV-sensitive strains of *Escherichia coli* to be isolated (Hill, 1958; Howard-Flanders & Theriot, 1962). This lack of success in isolating excision-defective mutants suggested either that excision repair contributes so little to the overall repair capacity of *D. radiodurans*, compared with recombination repair, that the difference in UV-sensitivity

Abbreviations: MMS, methyl methanesulphonate; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; MTC, mitomycin C.

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between excision-deficient and excision-proficient strains is not sufficiently large to allow the mutant to be isolated, or that mutation to give the excisionless phenotype is a very rare event.

A mitomycin C (MTC)-sensitive, UV-resistant mutant of *D. radiodurans* (strain 302, mutant in *mtcA*) has been isolated (Moseley & Copland, 1978) that is hypermutable by MNNG (Tempest & Moseley, 1978, 1980). On the basis that mutation to the excisionless phenotype is a rare event, it was decided to increase the chance of isolating such mutants by treating strain 302, rather than the wild-type, with MNNG. Having obtained putative excision-deficient mutants, i.e. displaying an UV-sensitive, methyl methanesulphonate (MMS)-resistant phenotype, the *mtcA* mutation could then be removed by transforming the double mutant with DNA from a strain wild-type for *mtcA* and selecting for MTC resistance (Moseley & Copland, 1978).

Several UV-sensitive, excisionless strains were isolated. However, when the *mtcA* gene in each was replaced by its wild-type allele all the mutants became UV-resistant. The resolution of this problem provides evidence for the existence of two, alternative, excision pathways in *D. radiodurans*.

METHODS

Bacteria. The strains of *Deinococcus radiodurans* used were all derivatives of *D. radiodurans* R_1 (Anderson *et al.*, 1956). They were: 302, mutant in *mtcA* and sensitive to MTC; 301, mutant in *mtcA* and *uvsA* and sensitive to MTC and UV radiation. Both 301 and 302 were sensitive on agar to 4 μg rifampicin ml^{-1}. *Deinococcus radiodurans* (Krase), resistant to 100 μg rifampicin ml^{-1} was used as a source of transforming DNA (Tirgari & Moseley, 1980).

Media. TGY medium for growth contained 5 g Bactotryptone (Difco), 1 g glucose and 3 g yeast extract (Difco) in 1 l distilled water. TGY agar was made by solidifying this medium with 15 g Bactoagar l^{-1}. Phosphate buffer (0·67 M, pH 7·0) for washing bacteria and suspending them for irradiation contained 4·73 g Na_2HPO_4 and 4·54 g KH_2PO_4 in 1 l distilled water.

Growth of bacteria. Exponential phase cultures were obtained by diluting samples from 18 h cultures into 20 ml amounts of TGY medium, in 250 ml conical flasks, to give a turbidity of about 0·08 in a nephelometer (EEL, Halstead, Essex) with an orange filter. The cultures were incubated with shaking at 30 °C until they reached an appropriate turbidity. A turbidity of 0·30 was equivalent to $1·0 \times 10^8$ viable units ml^{-1}.

Mutagenesis. To 10 ml of an exponential phase culture of *D. radiodurans* 302 growing at 30 °C and containing $1·3 \times 10^8$ viable units ml^{-1}, 0·2 ml MNNG in phosphate buffer (1 mg ml^{-1}) was added to give a final concentration of 20 μg MNNG ml^{-1} and the culture was reincubated. At intervals up to 120 min two 0·1 ml samples were removed, one diluted and plated on TGY agar to measure loss of viability, colonies from surviving bacteria being counted after 2 to 3 d at 30 °C, while the other sample was diluted into 20 ml TGY medium and incubated for 18 h at 30 °C with shaking. This diluted the MNNG to below the MIC and consequent growth allowed recessive mutations to segregate.

Isolation of mutants. Samples from mutagenized cultures were diluted in TGY medium and 0·1 ml amounts plated on TGY agar to give about 80 colonies per plate after incubation at 30 °C for 2 d. Two replicate copies of each plate were made on TGY agar, one copy being irradiated with a UV dose of 500 J m^{-2}. Both copies were incubated at 30 °C for 24 h and colonies that grew only on the unirradiated copy were tested individually for sensitivity to UV radiation. Initially, putative UV-sensitive clones were streaked in a paralleled array across the surfaces of TGY plates and increasing doses of UV radiation applied along the streaks. After incubation at 30 °C for 1 to 2 d those clones more sensitive to UV radiation than strain 302 were clearly distinguishable and irradiation survival curves were obtained.

Irradiation survival curves. Bacteria from exponential phase cultures containing about $1·0 \times 10^8$ viable units ml^{-1} were centrifuged, washed and resuspended in phosphate buffer at the same concentration. For UV irradiation a 5 ml sample of the washed suspension in a 9 cm Petri dish was irradiated at a distance of 40 cm from an Hanovia germicidal lamp, the suspension being agitated by a magnetic stirrer. The incident dose rate was 1·05 J m^{-2}. Gamma irradiation was carried out in a ^{60}Co source at a dose rate of 3·0 krad min^{-1}. Samples of the washed bacterial suspension (3 ml) were irradiated, oxygen being bubbled through the suspension during irradiation.

At intervals 0·1 ml samples from the populations being UV or γ irradiated were diluted in TGY medium and 0·1 ml samples spread on TGY agar plates. Colonies were counted after incubation at 30 °C for 3 to 4 d.

Resistance to methyl methanesulphonate. The bacteria from 10 ml amounts of exponentially growing cultures containing about 10^8 viable units ml^{-1} were centrifuged, washed and resuspended in 10 ml quantities of phosphate buffer and placed in 250 ml conical flasks. MMS (50 μl) was added to each flask and the suspensions incubated for 50 min at 30 °C. Appropriate dilutions of the suspension (0·1 ml) were spread on TGY plates and colonies from surviving bacteria were counted after incubation at 30 °C for 2 to 3 d.

Assay for sensitivity or resistance of cultures to alkylation mutagenesis. Wild-type *D. radiodurans* is very resistant to alkylation mutagenesis while strain 302 is very sensitive. The two cultures were used as controls in an assay for sensitivity or resistance to ethyl methanesulphonate (EMS) mutagenesis. One drop (approximately 0·02 ml) of EMS was added to one of two 0·8 ml cultures in exponential phase growth, the second culture acting as a non-treated control. After 45 min incubation at 30 °C 0·1 ml from each culture was added to 10 ml amounts of sterile TGY in 250 ml flasks and the cultures incubated for 18 h. Samples of the undiluted cultures (0·1 ml) were spread on TGY agar plates containing 10 μg rifampicin ml^{-1} to assay the number of rifampicin-resistant mutants and, when necessary, 0·1 ml amounts of appropriately diluted culture on TGY agar plates to assay viable numbers. Colonies were counted after 3 d incubation at 30 °C. Using this protocol the frequency of rifampicin-resistant mutants in an EMS treated population of wild-type *D. radiodurans* was $2{\cdot}0 \times 10^{-7}$ compared with a spontaneous frequency of $5{\cdot}2 \times 10^{-8}$, i.e. a 3·8-fold increase. For strain 302 the EMS induced frequency was $1{\cdot}5 \times 10^{-4}$ compared with a spontaneous frequency of $1{\cdot}0 \times 10^{-7}$, i.e. a 1500-fold increase. In order to score clones as resistant or sensitive it was unnecessary to measure viable numbers if the cultures were turbid since resistant clones gave 10 to 50 colonies per 0·1 ml undiluted culture and sensitive clones gave 1000 to 5000 colonies on rifampicin agar.

To test for UV mutability. Exponential phase cultures were prepared for UV radiation by centrifugation and resuspension into phosphate buffer. A 0·1 ml sample of unirradiated suspension was added to 10 ml sterile TGY in a 250 ml flask as an unirradiated control. A 5 ml portion of the suspension was irradiated with a suitable dose of UV and 0·1 ml amounts added to 10 ml quantities of TGY in 250 ml flasks. All the cultures were incubated for 48 h and 0·1 ml amounts of suitable dilutions were spread on TGY plates for viable counts and on TGY plates plus 10 μg rifampicin ml^{-1} for counts of rifampicin-resistant mutants.

Preparation of transforming DNA and the transformation procedure. These have been described previously in detail (Moseley & Copland, 1978). The selection of bacteria transformed from UV sensitivity to resistance was carried out using a dose of 540 J m^{-2} and of bacteria transformed from MTC sensitivity to resistance by plating on TGY agar containing MTC (Sigma) at a concentration of 0·05 μg ml^{-1}.

Measurement of pyrimidine dimer excision from the DNA of UV irradiated D. radiodurans. A 25 μl sample of an 18 h culture of *D. radiodurans* and 125 μCi tritiated thymidine [specific activity 27 Ci $mmol^{-1}$ (0·99 TBq $mmol^{-1}$), 1 mCi ml^{-1} (37 MBq ml^{-1})] were added to 2·5 ml TGY medium and incubated with shaking at 30 °C for 18 h. The labelled bacteria were centrifuged, resuspended in 5 ml TGY at 30 °C and incubated for 1 h at 30 °C. The culture was centrifuged, the bacteria resuspended in 2·5 ml phosphate buffer and UV irradiated with a dose of 360 J m^{-2}. The irradiated suspensions were centrifuged, the bacteria resuspended in 2·5 ml TGY at 30 °C and incubated in 0·5 ml amounts in microcentrifuge tubes. At varying times excision was terminated by adding 0·1 ml calf thymus DNA (5 mg ml^{-1}) and 0·6 ml ice-cold 12·5% TCA to each tube. The tubes were held on ice for 10 min, centrifuged and the precipitate washed with ice-cold 5% TCA and then with ice-cold ethanol. The pellets obtained from centrifugation were dried at 50 °C and resuspended in 0·2 ml 98% formic acid. The suspensions were transferred to 1 ml freeze-drying ampoules which were then sealed and heated to 175 °C for 1 h. The brown hydrolysates were spotted on to Whatman 3 MM chromatography paper, dried and run for 18 h in butanol/acetic acid/water (18:12:30, by vol.). The paper was dried, cut into 1 cm strips and each strip placed in a scintillation vial containing 0·5 ml water to dissolve the labelled nucleotides. Scintillant (4·5 ml) (NE 250, Nuclear Enterprises, Edinburgh) was added to each vial and the radioactivity assayed in a scintillation counter.

The percentage of thymine present in thymine-containing pyrimidine dimers was calculated from the ratio of counts formed in the dimer peak and the thymine peak.

RESULTS

Isolation of putative excision deficient mutants

Incubation of strain 302 with 20 μg MNNG ml^{-1} for 120 min did not reduce the viability of the culture. After allowing treated cells to segregate recessive mutations in liquid culture before plating, resulting colonies were replicated and the newly replicated plates UV irradiated. Of about 6000 colonies six were identified as sensitive to UV radiation after the second screening procedure. These were tested for their resistance to the lethal action of MMS, using wild-type *D. radiodurans* and strain 301 as controls for MMS resistance and sensitivity, respectively. The survival level of the wild-type after 50 min incubation in 5 μl MMS per ml of bacterial suspension was $3{\cdot}4 \times 10^{-1}$ and for 301 was $< 7 \times 10^{-7}$. Three of the six mutants, strains UVS9, 25 and 78, were resistant to MMS, having survival levels of $7{\cdot}2 \times 10^{-1}$, $7{\cdot}7 \times 10^{-1}$ and $3{\cdot}3 \times 10^{-1}$, respectively.

The UV and γ radiation survival curves of these three strains are shown in Fig. 1 and an

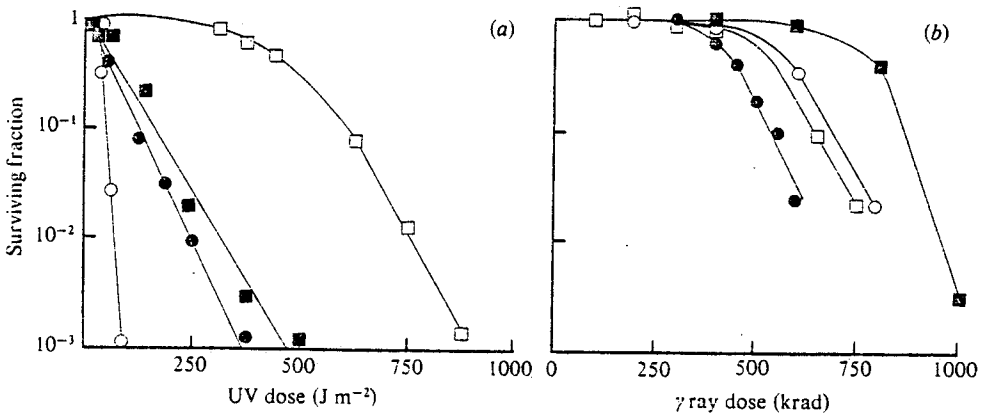

Fig. 1. (*a*) UV and (*b*) gamma irradiation survival curves of strains UVS9 (●), UVS25 (■) and UVS78 (○) compared with those of the wild-type strain of *Deinococcus radiodurans* (□).

Table 1. *Analysis of survival curves of strains UVS9, UVS25 and UVS78 of D. radiodurans*

The results are derived from Fig. 1.

	UV radiation				Ionizing radiation			
Strain	Intercept	1/e	D37	Ratio D37 wild-type (570 J m^{-2})/ D37 mutant	Intercept	1/e	D37	Ratio D37 wild-type (600 krad)/ D37 mutant
UVS9	0	54	54	10·5	375	25	400	1·5
UVS25	0	70	70	8	815	25	840	0·7
UVS78	33	8	40	14	695	25	585	1·03

analysis of the curves in Table 1. Strains UVS9 and 25 have no shoulder to their UV survival curves and an exponential inactivation slope almost equal to that of the wild-type, i.e. the new mutations have increased the sensitivity of strain 302 causing complete loss of the shoulder. UVS78 has a small shoulder to its UV survival curve, but the exponential loss of viability is much more sensitive than that of the wild type. The γ-ray survival curves show UVS25 to be more resistant, UVS78 to be as resistant and UVS9 to be slightly more sensitive, than the wild-type.

Transformation to UV resistance of each strain with DNA from the others

To show whether the new mutations introduced into strain 302, to create the UV sensitive strains UVS9, 25 and 78, are in the same or different genes each of the strains was incubated with DNA from each of the strains and the wild-type made resistant to rifampicin and selection made independently for UV-resistant and rifampicin-resistant transformants. The results are shown in Table 2. Strains UVS78 and UVS9 gave much higher frequencies of transformation for both markers than UVS25, for reasons that have not been investigated. However, it is clear that UVS78 could be transformed to UV resistance equally well using DNA from the wild-type, UVS9 and UVS25 and that UVS9 could be transformed to UV resistance using DNA from UVS25 and UVS78, although not as well as by DNA from wild-type. These results indicate that the mutations creating the strains UVS9, UVS25 and UVS78 are in different genes and these were designated *uvsC*, *uvsD* and *uvsE*, respectively.

Removal of the mtcA mutation from each of the UV sensitive strains

Since the mutations leading to UV sensitivity were induced in strain 302 which had an existing mutation in *mtcA*, conferring sensitivity to MTC, this was removed by incubating each

Table 2. *Transformation frequencies for rifampicin resistance and UV resistance in the wild-type and mutants UVS9, UVS25 and UVS78 of D. radiodurans incubated with DNA isolated from each of the strains*

Rifampicin resistance and UV resistance were tested 5 h and 9 h, respectively, after transformation.

	Recipient:			
Donor DNA	Wild-type	UVS9	UVS25	UVS78
		(*a*) *Rifampicin resistance*		
Wild-type	$5{\cdot}0 \times 10^{-3}$	$7{\cdot}7 \times 10^{-5}$	$1{\cdot}5 \times 10^{-4}$	$1{\cdot}6 \times 10^{-4}$
UVS9	$2{\cdot}5 \times 10^{-3}$	$3{\cdot}2 \times 10^{-5}$	$7{\cdot}9 \times 10^{-6}$	$7{\cdot}1 \times 10^{-4}$
UVS25	$1{\cdot}3 \times 10^{-3}$	$1{\cdot}7 \times 10^{-4}$	$8{\cdot}3 \times 10^{-6}$	$1{\cdot}7 \times 10^{-4}$
UVS78	$2{\cdot}5 \times 10^{-3}$	$7{\cdot}3 \times 10^{-5}$	$2{\cdot}3 \times 10^{-5}$	$2{\cdot}0 \times 10^{-4}$
		(*b*) *UV resistance*		
Wild-type	NA	$3{\cdot}2 \times 10^{-4}$	$4{\cdot}0 \times 10^{-5}$	$2{\cdot}3 \times 10^{-4}$
UVS9	NA	$<4{\cdot}7 \times 10^{-6}$	$7{\cdot}5 \times 10^{-5}$	$1{\cdot}8 \times 10^{-4}$
UVS25	NA	$2{\cdot}4 \times 10^{-4}$	$5{\cdot}2 \times 10^{-6}$	$3{\cdot}6 \times 10^{-4}$
UVS78	NA	$3{\cdot}9 \times 10^{-5}$	$1{\cdot}1 \times 10^{-4}$	$<1{\cdot}7 \times 10^{-7}$

NA, Not applicable.

of the new strains UVS9, 25 and 78, with DNA from the wild-type and selecting for transformants growing on TGY agar containing 0·05 μg MTC ml^{-1}. Transformation frequencies for UVS9, 25 and 78 were $1{\cdot}0 \times 10^{-2}$, 5×10^{-5} and $1{\cdot}0 \times 10^{-2}$, respectively. Two hundred MTC-resistant transformants of each of strains 9 and 25 and 347 MTC-resistant transformants of strain 78 were tested for their sensitivity to UV radiation by irradiating streaks of them on TGY agar with 500 J m^{-2}. All were resistant to UV radiation. Transformants of UVS9, UVS25 and UVS78 obtained in this way and having the MTC- and UV-resistant phenotype were given the strain description of 91, 251 and 781, respectively.

Isolation of UV-resistant transformants of strains UVS9, 25 and 78 and their phenotype with respect to mitomycin

Strains UVS9, 25 and 78 were incubated with DNA from the wild-type and UV-resistant transformants isolated. These were then tested for resistance or sensitivity to MTC by streak testing on TGY agar containing 0·05 μg MTC ml^{-1}. For UVS9 58% (35 of 60 colonies tested) of the UV resistant transformant colonies were also MTC-resistant, for UVS25, 56% (56 of 100) and for UVS78, 54% (159 of 295). As a control UVS78 was incubated with DNA from strain 301 and UV-resistant transformants were isolated. All were MTC sensitive since the *mtcA* mutation is common to both UVS78 and 301 and the wild-type allele cannot be substituted for the mutant one. Six UV-resistant, MTC-sensitive transformants of UVS78 were tested for resistance or sensitivity to alkylation-induced mutagenesis. All were sensitive. Six out of six UV-resistant, MTC-resistant clones were resistant to such mutagenesis.

To check the phenotypic effect of each of the mutations *uvsC*, *uvsD* and *uvsE* in a wild-type background UV survival curves were obtained for UV-resistant transformants of UVS9, 25 and 78 that were also MTC-resistant and resistant to mutation by alkylating agents by restoration of the wild-type allele at *mtcA* (Fig. 2). Six of each type were tested. Although there was variation in UV resistance in each group of six, the six transformants of UVS25 were more resistant than the transformants of UVS9 which in turn were more resistant than those of UVS78. The survival curve plotted from the average values for the six UVS9 transformants was identical to that of the wild-type.

Excision of pyrimidine dimers

The UV-resistant wild-type and strain 302 excised 50% of the pyrimidine dimers induced by 360 J m^{-2} UV radiation in about 18 min while the UV-sensitive double mutants UVS9, UVS25

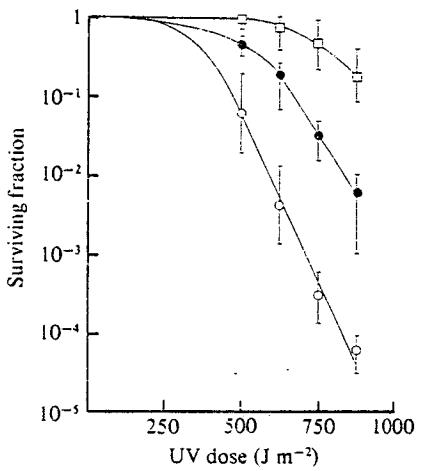

Fig. 2. UV irradiation survival curves of six clones each of strains UVS9, UVS25 and UVS78 transformed with DNA of the wild-type and selected for UV and MTC resistance. These strains were designated 91 (●), 251 (□) and 781 (○), respectively. The average values for each group of six are plotted, the bars representing the range within which individuals in each group fell.

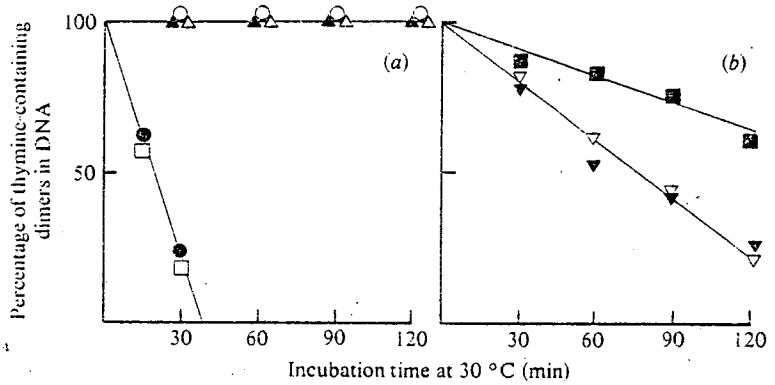

Fig. 3. Rate of loss of thymine containing pyrimidine dimers from the DNA of strains of *Deinococcus radiodurans* irradiated with 360 J m^{-2} UV radiation and incubated in TGY broth (*a*) wild-type (●), the single mutant, 302 (□), and the double mutants UVS9 (△), UVS25 (▲) and UVS78 (○); (*b*) the single mutants 91 (▼), 251 (▽) and 781 (■).

and UVS78 were unable to excise any dimers during 120 min incubation (Fig. 3*a*). The UV-resistant single mutants 91, 251 and 781 excised dimers, but more slowly than the wild-type, 91 and 251 releasing 50% in about 80 min while 781 had released only about 35% at 120 min (Fig. 3*b*).

UV non-mutability of excision-defective strains

The frequencies of rifampicin-resistant mutants in populations of bacteria grown from UV- and non-irradiated suspensions of UVS9, 25 and 78 were calculated from experimental data. The doses of UV administered were 63 J m^{-2} for UVS9 and UVS78 and 126 J m^{-2} for UVS25. For UVS9, the frequency of rifampicin-resistant mutants from an unirradiated population was 4×10^{-7} and in an irradiated one, 6×10^{-7}. For UVS25, the corresponding values were 8×10^{-7} and 6×10^{-7} and for UVS78 they were 2×10^{-6} and $1{\cdot}2 \times 10^{-6}$. Thus there was no evidence for UV-induced mutation.

Table 3. *Summary of results for UVS78*

Strain	Genotype *mtcA*	Genotype *uvsE*	Phenotype* UV	MTC	Alk. mut	Dimer excision
Wiid-type	+	+	R	R	R	+
302	−	+	R	S	S	+
	MNNG mutagenesis					
UVS78	−	−	S	S	S	−
	Transformation with wild-type DNA					
	Select for MTC^R					
731	+	−	R	R	R	+
	Select for UV^R					
781 and	+	−	R	R	R	+
302	−	+	R	S	S	+

* Alk. mut denotes alkylation mutagenesis: R denotes resistant and S denotes sensitive.

The results for the isolation of UVS78 and its transformation to other genotypes are summarized in Table 3. The results for UVS9 and 25 were similar.

DISCUSSION

In order to increase the chance of isolating excision-defective mutants of *D. radiodurans*, it was decided to mutagenize a population of strain 302 rather than wild-type because the former yields fifty times as many MNNG-induced mutants as the latter. It was assumed that excision-defective strains would be generated by single mutations in genes analogous to the *uvrA*, *B* and *C* genes of *Escherichia coli*. It was also assumed that the *mtcA* gene which had been demonstrated to have a role in the resistance of the wild-type to alkylation mutagenesis and MTC-induced lethality did not contribute to UV resistance since a strain mutant in this gene has the same UV resistance as the wild-type. This implied that the mutant *mtcA* gene could be replaced by its wild-type allele in putative excision defective strains in order to study the effect of any newly induced mutation.

Three strains were isolated having some of the properties of excision-deficient mutants, viz. UV sensitivity and MMS resistance, and were subsequently shown to be unable to excise UV-induced thymine-containing pyrimidine dimers. The three newly induced mutations in the three strains UVS9, 25 and 78 were different from each other on the basis that DNA from each of the strains could transform both of the others to UV resistance and so the respective genes were designated *uvsC*, *D* and *E*. The genes *uvsA* and *B* have already been assigned, mutations in these genes causing sensitivity to UV radiation in strains 303 and 263, respectively (Moseley & Copland, 1978). However, when the mutant *mtcA* gene in each of UVS9, 25 and 78 was replaced by its wild-type allele then strains possessing only one of the mutations *uvsC*, *D* or *E* regained resistance to UV radiation, i.e. mutations in *uvsC*, *D* or *E* could only confer UV sensitivity in strains already mutant in *mtcA* or vice versa. Thus the wild-type genes *uvsC*, *D* or *E* conferred UV resistance on a strain in the presence of a mutant *mtcA* gene (strain 302) and conversely a wild-type *mtcA* gene conferred UV resistance on strains mutant in *uvsC*, *D* or *E* (strains 91, 251 and 781, respectively). The UV-resistant strains 91, 251 and 781 carrying single mutations in *uvsC*, *D* and *E*, respectively, excised thymine-containing pyrimidine dimers, but at varying rates and in all cases slower than that of the wild-type and 302. The lower rates in these mutants is

taken as a reflection of the relative importance of the *uvsC, D, E* pathway compared with that governed by the *mtcA* gene, viz. the *uvsC, D, E* pathway excises dimers at a faster rate than does the *mtcA* pathway and since on its own it excises pyrimidine dimers at the same rate as when both pathways are operating must play a major role in the wild-type. However, a maximum rate of dimer excision does not necessarily give maximum survival since the strains mutant in *uvsD* (UVS25) had slower excision rates than wild-type, but a greater resistance to UV radiation.

The evidence for two excision pathways offers an explanation for the prior lack of success in isolating excision-deficient mutants since it is now clear that two independent mutations are required for the UV-sensitive phenotype to be displayed, a result fortuitously gained by choosing to treat with MNNG a strain already mutant in one of them. The possession by an organism of two excision pathways either one of which is capable of conferring wild-type resistance to UV radiation has not been reported previously. Two UV endonucleases, I and II, have been isolated from wild-type *Micrococcus luteus* (Riazuddin & Grossman, 1977*a*, *b*), but both are apparently required in a cell to confer UV resistance (Riazuddin *et al.*, 1977). Thus a strain of *M. luteus* lacking both endonucleases and one lacking only endonuclease I were UV-sensitive while a strain lacking only endonuclease II could not be isolated by transformation with wild-type DNA of the strain lacking both. If the presence of endonuclease I alone had conferred UV resistance its selection would have been straightforward.

None of the mutations *uvsC, D* or *E* has been shown to be closely linked to *mtcA* since UV resistant transformants selected from the double mutants UVS9, 25 and 78 incubated with wild-type DNA fell approximately equally into two groups, viz. those which were MTC-sensitive, presumably having the mutant genes *uvsC, D* or *E* replaced by the wild-type allele, giving strains isogenic to 302, and those which were resistant to MTC and alkylation mutagenesis, presumably having the mutant *mtcA* gene replaced by its wild-type allele, but containing singly the mutant genes *uvsC, D* or *E* (strains 91, 251 and 781, respectively). Thus, for example, in UVS9 about 50% of the transformation events replaced the *mtcA* gene and 50% the *uvsC* gene and there was no marked skew to the UV-resistant MTC-resistant phenotype which one would expect if a substantial number of single transformation events replaced both genes. The values of 58, 56 and 54% for the proportion of transformants of UVS9, 25 and 78 having the UV-resistant MTC-resistant phenotype are assumed not to be significantly different from 50% in view of the numbers of clones screened.

Excision-deficient mutants of *E. coli* are much more mutable by UV light than the wild-type (Hill, 1965) and Witkin (1969) calculated that an unexcised dimer must be at least 500 times more likely to cause a mutation than an excised one, as a result of its passing along an error-prone repair pathway. *Deinococcus radiodurans* has been reported not to possess an error prone repair pathway for UV-induced damage (Sweet & Moseley, 1974, 1976) and this is confirmed by the lack of any induced mutation in excision-deficient mutants of *D. radiodurans*.

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Roles of the *uvsC*, *uvsD*, *uvsE*, and *mtcA* Genes in the Two Pyrimidine Dimer Excision Repair Pathways of *Deinococcus radiodurans*

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In *Deinococcus radiodurans*, the genes *uvsC*, *uvsD*, *uvsE*, and *mtcA* are all involved in the single-strand incision of UV-irradiated DNA, and mutations in at least two of them were required to produce an incisionless strain. One mutation must be in *mtcA* and one in *uvsC*, *uvsD*, or *uvsE*. Strains carrying single mutations in any one of the genes can incise DNA to the same extent as the wild-type strain. Neither the presence of EDTA nor the absence of protein synthesis affected the incision step. Strains deficient in DNA incision have greatly reduced DNA degradation after UV irradiation, and upon addition of chloramphenicol to the postirradiation medium, they do not undergo excessive DNA degradation as is seen in the wild-type strain and strains singly mutant in *uvsC*, *uvsD*, or *uvsE*. The strain singly mutant in *mtcA* also lacked chloramphenicol-enhanced DNA degradation and loss of viability but behaved similarly to the wild-type strain with respect to resumption of DNA synthesis and DNA degradation in the absence of chloramphenicol. It is proposed that two constitutive, cation-independent UV endonucleases are present in *D. radiodurans*: UV endonuclease α (the product of the *mtcA* gene), which incises in response to pyrimidine dimers, mitomycin C cross-links, bromomethylbenzanthracene adducts, and other alkylation damage, and UV endonuclease β (the product of the *uvsC*, *uvsD*, and *uvsE* genes), which incises only in response to pyrimidine dimers. Both endonucleases have associated exonuclease activity. The exonucleolytic activity associated with UV endonuclease α requires a UV-induced protein to terminate (or control) its activity, whereas the exonucleolytic activity associated with UV endonuclease β is slower acting and does not require the inducible terminator.

Deinococcus (formerly *Micrococcus*) *radiodurans* is the type species of a group of four red-pigmented, radiation-resistant bacteria that comprise one of the eight major groups of the eubacteria (5). Although *D. radiodurans* is extremely resistant to both the lethal and mutagenic effects of a variety of DNA-damaging agents (21), it has been shown to possess only two mechanisms for the repair of UV-irradiated DNA: excision repair (2) and recombination repair (14, 17). Excision repair of pyrimidine dimers in *D. radiodurans* can proceed through two distinct, equally efficient pathways, the removal of either by mutation not markedly affecting UV resistance (16). One pathway requires a functional *mtcA* gene, and the other pathway requires functional *uvsC*, *uvsD*, and *uvsE* genes. Only when both pathways are blocked by mutations are cells incapable of excising pyrimidine dimers. In this paper, the excision repair process is examined further in appropriate mutants to define the two excision repair pathways operating in the wild-type strain.

MATERIALS AND METHODS

Strains and growth conditions. The strains used and their relevant genotypes are noted in Table 1. All strains were grown in TGY medium (15), which contained 5 g of tryptone, 1 g of glucose, and 3 g of yeast extract per liter, at 30°C. Exponentially growing cultures were used for all experiments. Cell DNA was prelabeled when required by diluting an 18-h culture into TGY containing [^{3}H]thymidine (20 μCi ml^{-1}). The cultures were then grown for 18 h so that at the end of this period the cultures were still in exponential growth.

Irradiation of cultures. Cultures were washed twice and suspended in 67 mM phosphate buffer (pH 7.0) (22) at a concentration of ca. 10^7 viable units ml^{-1}. The cultures were UV irradiated at a distance of 30 cm from a Hanovia germicidal lamp (dose rate, 1.05 J m^{-2} s^{-1}). Cultures were agitated during irradiation. All the UV doses used allowed 100% of the irradiated bacteria to survive.

TABLE 1. List of strains of *D. radiodurans* used

Strain	Genes carrying mutations	Reference
R_1 (wild type)		1
302	*mtcA*	15
uvs9	*mtcA uvsC*	16
uvs25	*mtcA uvsD*	16
uvs78	*mtcA uvsE*	16
91	*uvsC*	16
251	*uvsD*	16
781	*uvsE*	16

Measurements of single-strand breaks. Labeled cultures were resuspended in TGY and incubated for 1 h. When appropriate, incubation was continued for a further 30 min in TGY containing chloramphenicol (15 μg ml^{-1}) or 20 mM EDTA. Samples (0.5 ml) were withdrawn and irradiated, in some cases in the presence of chloramphenicol or EDTA. UV absorption by these compounds under the irradiation conditions used was negligible. Preparation of bacteria for lysis onto alkaline sucrose gradients, construction of gradients, and conditions of centrifugation have been described in detail previously (22).

After centrifugation, the gradients were drained by using a fine capillary inserted through the gradient to the bottom of the tube. Fractions were collected by pumping onto strips of Whatman 3 MM chromatography paper, and the radioactivity of fractions was determined as described previously (22).

Measurement of DNA degradation. Labeled cells were resuspended in TGY, and growth was continued for an hour to exhaust intracellular pools of radioactivity. The cultures were UV irradiated and resuspended in TGY (in some instances containing chloramphenicol). Two 20-μl and one 100-μl samples were withdrawn at various times. The 100-μl samples were placed in microcentrifuge tubes containing 50 μl of a concentrated suspension of nonradioactive *D. radiodurans* in 67 mM phosphate buffer (pH 7.0), which ensured complete sedimentation of the labeled cells upon centrifugation. A total of 100 μl of the supernatant was removed, and the radioactivity was determined by using dioxan-based scintillant (Nuclear Enterprises; NE 250). The two 20-μl samples were placed on 1.8-cm Whatman no. 1 filter disks which were dried, washed twice in 5% trichloroacetic acid and twice in absolute ethanol, and dried, and the radioactivity was determined by using toluene-based scintillant (Nuclear Enterprises; NE 233). The amount of radioactivity measured on the disks was corrected to account for differences in counts obtained with the different scintillants. Label released into the medium was expressed as a percentage of that on the disks at zero time. DNA degraded is shown as the amount of label released into the medium.

Measurement of survival in the presence of chloramphenicol. Chloramphenicol (15 μg ml^{-1}) was added to the irradiated suspension of bacteria in 67 mM phosphate buffer immediately after irradiation. The bacteria were then suspended in TGY containing chloramphenicol and incubated with shaking at 30°C. Samples were withdrawn at various times, diluted, and plated on TGY agar. Colonies were counted after incubation for 4 days at 30°C.

Measurement of the UV-induced delay in DNA synthesis. Unlabeled cultures were irradiated and resuspended in TGY containing 40 μCi of [^{3}H]thymidine ml^{-1}, 15-μl samples were withdrawn at intervals and placed on 1.8-cm filter disks, and the radioactivity in trichloracetic acid-insoluble material was determined as described above.

RESULTS

Incision after UV irradiation. The production of single-strand breaks in the DNA of the wild-type strain after a sublethal dose of UV irradiation was compared with those in the UV-resistant strains, mutant in only one gene, and the UV-sensitive strains, mutant in two genes, one from each excision repair pathway (Fig. 1). The extent of incision in the singly mutant strains was approximately the same as in the wild-type strain. The UV-sensitive double mutants did not produce single-strand breaks in their DNA after irradiation. The inability of strain uvs78 to incise UV-irradiated DNA persisted during extended periods of incubation in TGY. Strains uvs9 and uvs25 showed a slight decrease in the molecular weight of their DNA upon incubation in TGY for 2 h. After sublethal UV doses which exceeded 300 J m^{-2}, the number of single-strand breaks began to rise, but not to the level in the wild-type strain (Fig. 2). The presence of chloramphenicol (15 μg ml^{-1}) or 20 mM EDTA added to the medium 30 min before irradiation did not inhibit the introduction of single-strand breaks (data not shown).

Effect of chloramphenicol on survival. When the wild-type strain was incubated after irradiation in TGY containing chloramphenicol, viability decreased with increasing times of incubation (Fig. 3). Strains carrying a single mutation in *uvsC*, *uvsD*, or *uvsE* showed a similar decline. However, the presence of chloramphenicol did not affect the viability of the strain singly mutant in *mtcA*. It was not possible to assess the effect of chloramphenicol upon the survival of the doubly mutant strains, because the doses of UV required to show the chloramphenicol effect exceeded those that were lethal to these strains.

DNA degradation. The rate and extent of DNA degradation after UV irradiation differed in all the strains from those in the wild-type strain (Fig. 4). Strains uvs9, uvs25, and uvs78 showed a marked reduction in the amount of DNA degraded, with a residual rate of DNA degradation that was dose independent. At high UV doses, strain 302 showed a slightly slower initial rate of degradation than the wild-type strain but a greater extent. The initial rate was further reduced in strains 91, 251, and 781, and in some instances the biphasic nature of degradation was lost. The addition of 10 mM EDTA to the

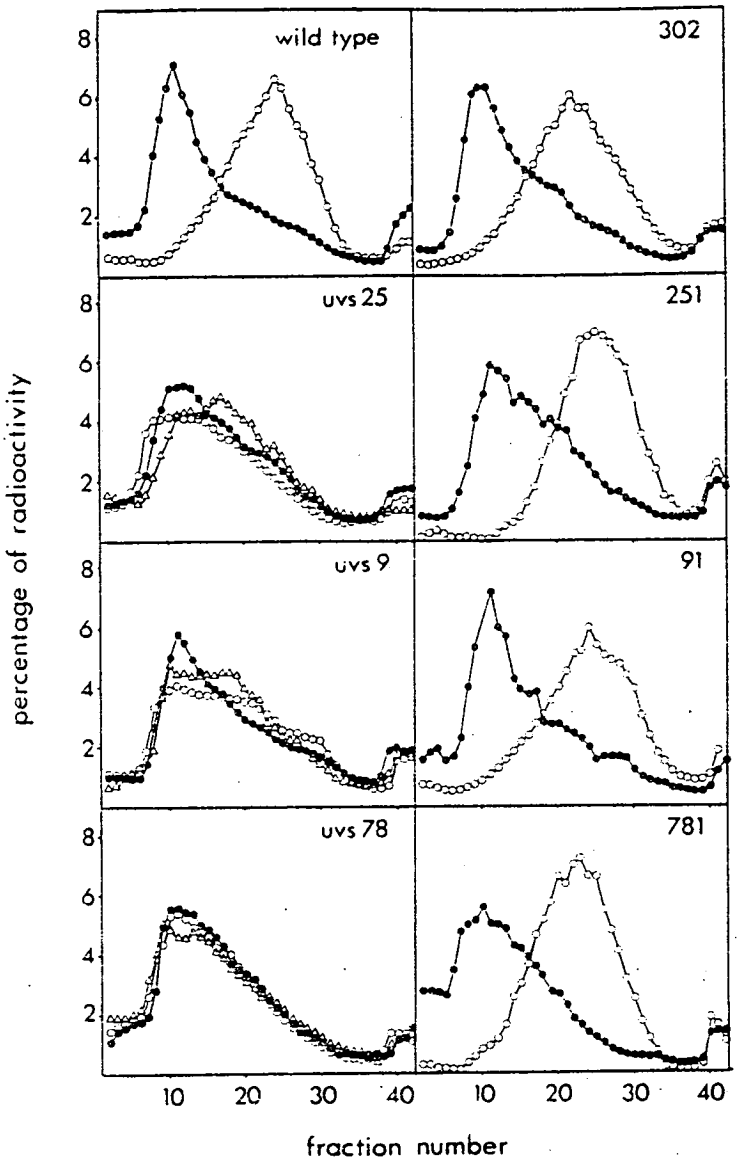

FIG. 1. Sedimentation profiles of DNA from *D. radiodurans* strains after UV irradiation (120 J m^{-2}). Spheroplasts of bacteria labeled with [^{3}H]thymidine were lysed directly onto alkaline sucrose gradients and centrifuged. Symbols: ●, undamaged DNA; ○, DNA after UV irradiation; △, DNA after UV irradiation and 2 h of incubation in TGY at 30°C.

postirradiation medium eliminated all degradation (data not shown).

The effect of chloramphenicol on DNA degradation. The addition of chloramphenicol to the postirradiation medium caused an increase in the rate and extent of DNA degradation in the wild-type strain if the UV dose exceeded about 30 J m^{-2}. Between 30 and 150 J m^{-2}, the final amount of DNA degraded was proportional to the UV dose. Above this dose, the degradation proceeded to completion at a dose-independent rate. A delay of 30 min after irradiation before the addition of chloramphenicol was sufficient to eliminate the enhancing effect of chloramphenicol on degradation for UV doses up to 360 J m^{-2} (data not shown). Chloramphenicol also enhanced DNA degradation in strains 91, 251, and 781 in the same manner as the wild type, but not in the doubly mutant strains uvs9, uvs25, and uvs78 or in strain 302 (Fig. 5). The addition of 10 mM EDTA to the postirradiation medium prevented all DNA degradation in the presence of

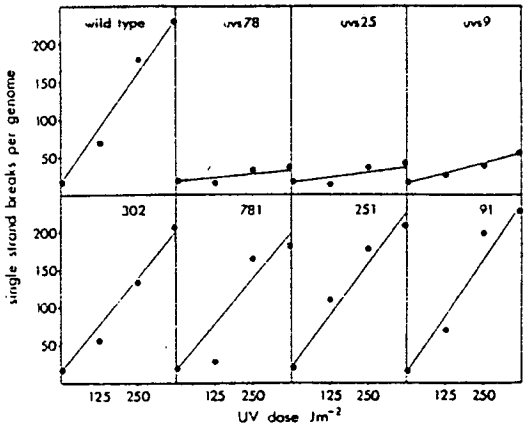

FIG. 2. The number of DNA single-strand breaks produced by *D. radiodurans* strains after UV irradiation. Values were obtained from the number of average molecular weights calculated directly with labeled λ and T4 DNA as calibration standards. The molecular weight of one genome is taken as 2.0×10^9 (9).

chloramphenicol (data not shown).

Delay in DNA synthesis. The delay in DNA synthesis after UV doses of 180 and 360 J m^{-2} was ca. 30 and 60 min, respectively, in the wild-type strain and also in strain 302 (Fig. 6). Strains that are incision deficient did not resume the control rate of uptake of label into DNA and retained the block to replication throughout the duration of the experiment. However, some residual incorporation of label was present in these strains. Strains 91, 251, and 781 incorporated label at a reduced rate compared with the wild type at equivalent doses.

DISCUSSION

Incision of DNA. The incision of DNA in *D. radiodurans* in response to UV irradiation is not affected by single mutations in genes *uvsC*, *uvsD*, *uvsE*, or *mtcA*. However, strains that are doubly mutant, *uvsC mtcA*, *uvsD mtcA*, or *uvsE mtcA*, are unable to incise their DNA after UV irradiation and consequently are unable to remove pyrimidine dimers from DNA. Two UV endonucleases are therefore postulated to exist in *D. radiodurans* which both incise DNA in response to pyrimidine dimers: UV endonuclease α, the product of the *mtcA* gene, and UV endonuclease β, the product of the *uvsC*, *uvsD*, and *uvsE* genes. The *uvsC*, *uvsD*, and *uvsE* genes have previously been shown to be unlinked (16) and may be comparable to the *uvrA*, *uvrB*, and *uvrC* genes of *Escherichia coli* in that they may code for discrete components of a multienzyme complex. Both UV endonucleases produce individually in the appropriate mutants approximately the same number of incisions as when both are present in the wild-type strain. In view of this, it would seem that the quantity of available UV endonuclease may not be the limiting factor in the observed numbers of single-strand breaks, which represent about 1% of the pyrimidine dimers present immediately after irradiation. Perhaps some factor required by both UV endonucleases for their action is in limited supply or their access to the damage is restricted. Gene *mtcA* is known to be essential for incision of bromomethylbenzanthracene-DNA adducts and for the removal of O^6-methylguanine and mitomycin C adducts from DNA (22). UV endonuclease α would therefore have a broad substrate specificity comparable to that of the *E. coli uvrABC* endonuclease (19) and unlike the smaller-molecular-weight UV glycosylases of T4 or *Micrococcus luteus* which are specific for pyrimidine dimers. It has been suggested that *mtcA* codes for a glycosylase (22), and it is possible that the UV endonuclease α could be the combined action of a glycosylase and an apyrimidinic endonuclease. However, it would

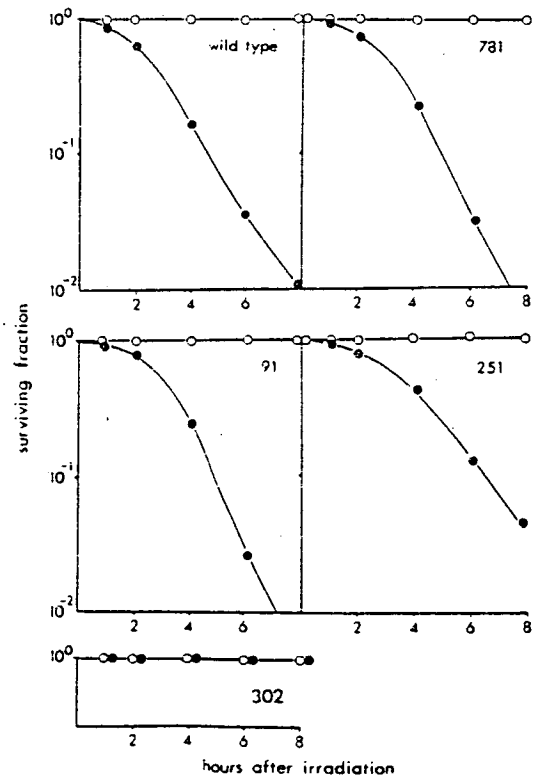

FIG. 3. Loss of viability in the presence of chloramphenicol. Cells exposed to sublethal doses of UV light were incubated in medium containing chloramphenicol (15 μg ml^{-1}). The surviving fraction was determined after incubation for various times at 30°C. Symbols: O, unirradiated cultures; ●, irradiated cultures (500 J m^{-2}).

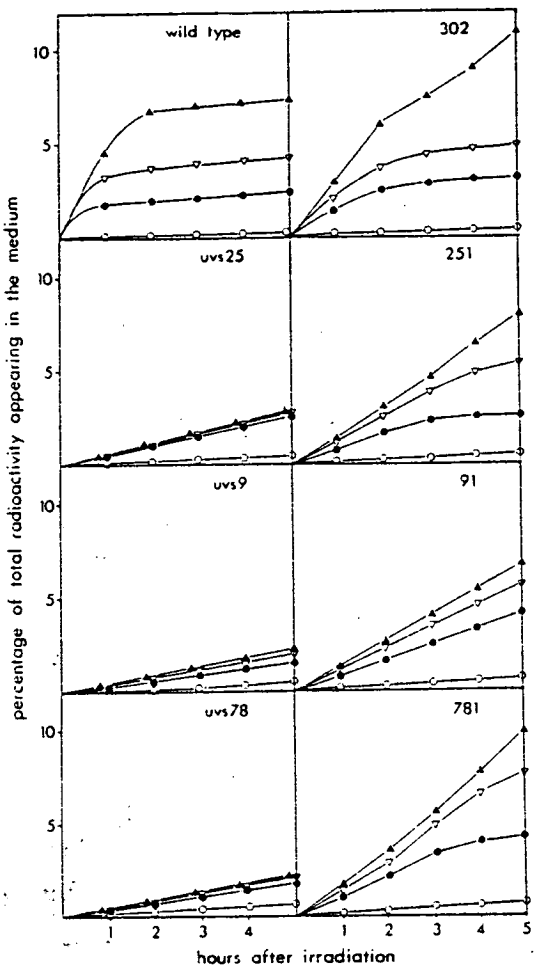

FIG. 4. Appearance of label in the medium after UV irradiation of *D. radiodurans* strains. Strains were grown for at least seven generations in the presence of [^{3}H]thymidine and then in medium without label. The cultures were irradiated and then incubated in TGY medium. The amount of radioactivity released into the medium was determined at various times. Symbols: ○, unirradiated cells; ●, 125 J m^{-2}; △, 250 J m^{-2}; ▲, 500 J m^{-2}.

be unusual for a glycosylase to have such a broad spectrum of substrates (11). Indeed both the α and β UV endonucleases may be the combined action of glycosylases and apyrimidinic endonucleases, and so the term UV endonuclease is used with that possibility in mind. However, the product of excision repair is known to be intact pyrimidine dimers which are part of short digonucleotides and not free thymine which indicates a *uvrABC* style of incision (2, 20). The ability of either UV endonuclease to act in the absence of protein synthesis suggests that both are constitutive or, if they are inducible, that the constitutive level is functionally adequate to cope with large amounts of damage. Both UV endonucleases act in the presence of EDTA, which indicates no divalent cation requirement, unlike the *E. coli uvrABC* endonuclease (19) but similar to the T4 (13) or *M. luteus* glycosylase and apyrimidinic endonuclease systems (6). EDTA has been shown to inhibit what must presumably be a different endonuclease, induced by butanol treatment of *D. radiodurans* (3).

DNA degradation. A period of DNA degradation followed the incision event. Evidence that the degradation is the result of excision repair is that DNA degradation is greatly reduced in incision-deficient strains. The amount of DNA degraded corresponded to ca. 20 nucleotides removed for each dimer present. This number is comparable to the numbers removed by excision repair in *E. coli* (8) and would be consistent with the notion that the degradation is confined to the dimer localities and is not a secondary event. The time during which the initial fast component of UV-induced DNA degradation was observed in wild-type *D. radiodurans* is consistent with the time required for removal of pyrimidine dimers from the DNA (16). DNA degradation was abolished by 10 mM EDTA, indicating a divalent cation requirement by the enzymes involved in the degradation (data not shown). In the singly mutant strains 91, 251, and 781, the rates of DNA degradation were lower than those of the wild type, but the quantity of DNA degraded was greater for particular UV doses. The rates are in general agreement with the rates of removal of thymine-containing dimers by these strains (16). The implication is therefore that, although these strains which possess only UV endonuclease α produce a comparable number of incisions to the wild type, the incisions produced are not acted upon by exonucleases in the same fashion as in the wild-type strain.

Effect of chloramphenicol on survival and DNA degradation. Inhibition of protein synthesis in the wild-type strain presumably prevented synthesis of a protein (an inducible "terminator") which is essential for attenuation of one of the exonucleases and thereby caused uncontroiled DNA degradation by the exonuclease and consequently cell death at UV doses above 120 J m^{-2}.

This would indicate either that the preinduced level of the protein is sufficient to control the exonuclease activity generated by a low UV dose or that the exonuclease only operates at higher UV doses. A similar enhancement of DNA degradation by chloramphenicol after UV irradiation is known in *E. coli* (18), and it has been suggested that normally the *recA* protein (24) or the ssb protein (12) would bind to DNA

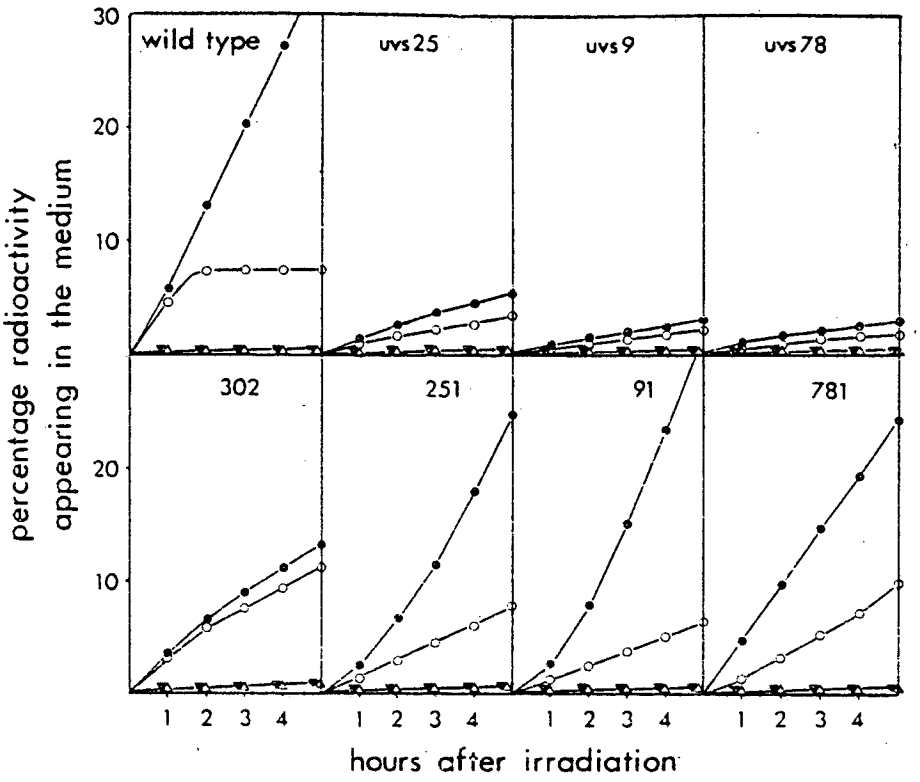

FIG. 5. Appearance of label in medium containing chloramphenicol after irradiation of *D. radiodurans* strains. Labeled cells were incubated in TGY containing chloramphenicol after UV irradiation. Symbols: ○, ▽, no chloramphenicol in the medium; ●, ▼, chloramphenicol (15 μg ml^{-1}) in the medium; ○, ●, irradiated cells (500 J m^{-2}); ▽, ▼, unirradiated cells.

after UV induction and prevent the progression of the *recBC* exonuclease along the DNA (23, 24). In the absence of protein synthesis and *recA* induction, the *recBC* exonuclease is free to degrade the chromosome in an uncontrolled manner after UV irradiation (4, 7). *D. radiodurans* strains lacking UV endonuclease β require post-UV protein synthesis to prevent uncontrolled DNA degradation and loss of viability, whereas it is not required in the doubly UV endonuclease-deficient mutants or in the mutant lacking UV endonuclease α (Fig. 3 and 4). The simplest explanation of this is that the exonuclease which requires the inducible terminator also requires the particular incision produced by UV endonuclease α as its substrate. Alternatively, it is possible that the inducible terminator is synthesized constitutively in strains lacking a functional *mtcA* gene (10), but it is difficult to imagine a simple mechanism whereby the gain of the *mtcA* α UV endonuclease by transformation (16) simultaneously releases control of the inducible terminator in such a way that its synthesis becomes constitutive.

An attractive overall explanation for these observations is that UV endonuclease α acts in a different manner from UV endonuclease β and therefore defines the manner of subsequent damage removal.

Resumption of DNA synthesis. The extended delay in DNA synthesis in strains lacking only UV endonuclease β (Fig. 6) is assumed to be due to the slower-than-wild-type excision by the activity associated with UV endonuclease α. Pyrimidine dimers would remain for longer periods in the DNA and thus block DNA replication. In the absence of both UV endonucleases in strains uvs9, uvs25, and uvs78, blocks to replication persist even longer owing to the failure of both excision repair pathways to remove pyrimidine dimers from the DNA.

It is therefore proposed that *D. radiodurans* possesses two constitutive, cation-independent UV endonucleases, at least one being a multiunit enzyme. Both produce incisions after UV irradiation that define the subsequent exonuclease action such that UV endonuclease α incisions are acted upon by an exonuclease which requires an inducible terminator, and UV endonuclease β incisions are acted upon by a slower-

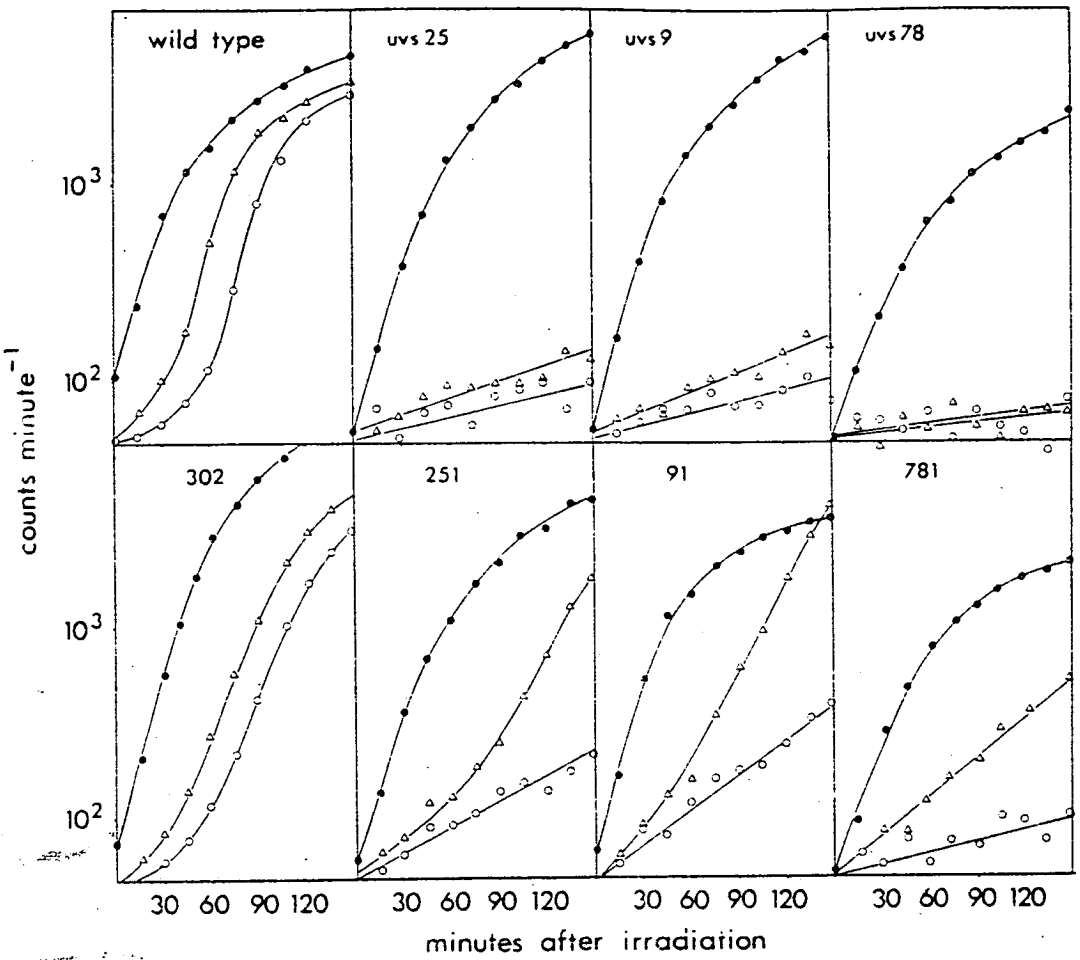

FIG. 6. Incorporation of [^{3}H]thymidine into trichloroacetic acid-insoluble form by irradiated *D. radiodurans* strains. Exponential-phase cultures were UV irradiated and then incubated in medium containing the label. Symbols: ●, unirradiated cultures; △, UV irradiated (200 J m^{-2}); ○, UV irradiated (400 J m^{-2}).

acting process which does not require the inducible terminator.

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